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(54) Title: GENE DELIVERY TO MUCOSAL EPITHELIUM FOR IMMUNIZATION OR THERAPEUTIC PURPOSES

(57) **Abstract**

Disclosed are compositions and method for transfecting mammalian mucosal epithelia with nucleic acid/cationic lipid complexes. The nucleic acid/cationic lipid complexes may be administered, for example, intranasally or directly into the lungs. The best results are obtained when the lipid mixed with the maximum amount of DNA that it can complex.

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Gene Delivery to Mucosal Epithelium for Immunization or Therapeutic Purposes

Background of the Invention

Field of the Invention

The present invention is in the fields of gene therapy and immunology. In particular, this invention is directed to methods of immunization and gene therapy using compositions comprising cationic lipids and polynucleotide molecules which code for immunogens or therapeutic genes, respectively. This invention is also directed to methods for producing polyclonal and monoclonal antibodies from genetically immunized animals.

Related Art

Traditional methods of immunization are achieved by injection of a mixture of antibodies which immunoreact with an invading pathogen (i.e., passive immunization), or by vaccination, which stimulates the immune system to produce pathogen-specific antibodies (i.e active immunization). Since foreign antibodies are cleared by the recipient, passive immunity confers only temporary protection. Vaccination confers longer-lasting active immunity.

In order to be effective, vaccination must generate humoral and/or cell-mediated immunity which will prevent the development of disease upon subsequent exposure to the corresponding pathogen. The pertinent antigenic determinants must be presented to the immune system in a manner that mimics a natural infection. Conventional vaccines may consist of inactivated virulent strains, or live-attenuated strains (Old *et al.*, *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, Blackwell Scientific Publications, 4th

edition, 1989). A general problem with using a vaccine consisting of whole virus is that many viruses (such as hepatitis B virus) have not been adapted to grow to high titre in tissue culture and thus, cannot be produced in sufficient quantity (*Id.*). In addition, the use of inactivated viruses present a potential danger of vaccine-related disease resulting from replication-competent virus may remain in the inoculum. Outbreaks of foot-and-mouth disease in Europe have been attributed to this cause (*Id.*). On the other hand, attenuated virus strains have the potential to revert to virulent phenotype upon replication in the vaccinee. This problem has been reported to occur about once or twice in every million people who receive live polio vaccine (*Id.*). Moreover, encephalitis can occur following measles immunization with attenuated virus (Roit, I.M. *Essential Immunology*, Blackwell Scientific Publications, Sixth Ed., 1988). Another disadvantage of using attenuated strains is the difficulty and expense of maintaining appropriate cold storage facilities (*Id.*). A major disadvantage associated with the use of live virus vaccines is that persons with congenital or acquired immunodeficiency risk severe infections. Such persons include children in developing countries who are often immunodeficient because of malnutrition and/or infection with viruses or parasites (*Id.*, Old *et al.*, *supra*).

As a result of recent advances in molecular biology and peptide synthesis, it is possible to produce purified viral proteins or synthetic peptides for use in immunoprophylaxis (Murphy *et al.*, "Immunization Against Viruses," in *Virology*, Fields *et al.*, Eds., Raven Press, New York, pp. 349-370, 1985). Purified antigens may be produced by synthesizing peptides which represent immunologically important domains of surface antigens of the pathogen. The synthetic peptide approach has been successfully used with an antigenic determinant of the foot and mouth disease virus (*Id.*). One problem with this approach is that the poor antigenicity of synthetic peptides has required the use of Freund's adjuvant to enhance the immune response in experimental animals (*Id.*). Since Freund's adjuvant cannot be used in humans,

an effective adjuvant for human use such as the saponins has been developed.

In addition, a single antigenic site may not be sufficient to induce resistance since large surface antigens usually contain several distinct immunological domains that elicit a protective humoral and/or cell-mediated response (Braciale *et al., J. Exp. Med. 153*:910-923 (1981); Wiley *et al., Nature 289*:373-378 (1981)). There may also be difficulties in stimulating an immunologic response to epitopes that are formed by noncontiguous parts of the linear protein molecule (Murphy, *et al., supra*). There is evidence that the majority of protein determinants are discontinuous and involve amino acid residues that are far apart in the primary amino acid sequence, but are brought into close juxtaposition by peptide folding (Roit, *supra*).

The alternative approach to preparing proteins for vaccines involves the use of cloned viral DNA inserted into a suitable vector to produce viral protein in prokaryotic or eukaryotic cells (Aldovini *et al., The New Vaccines, Technology Review*, pp. 24-31, January 1992). This approach, also, has several limitations. For example, one must devise suitable conditions for the optimal production of the recombinant protein of interest by the recombinant host cells. The protein product must be isolated and purified from the culture system, and obtained in sufficient quantities for use as a vaccine. Finally, it may be necessary to perform post-translational modifications of the purified protein (such as glycosylation and/or cleavage of a fusion protein).

An alternative to producing the recombinant antigen *in vitro* is to introduce nucleic acid sequences coding for the antigen into the cells of the vaccinee. In this way, the antigen is produced *in vivo* by the vaccinee's cells and provokes the immune response. Tang *et al.* (*Nature 356*:152-154 (1992)) have shown that it is possible produce an immune response to human growth hormone protein in mice by propelling gold microprojectiles coated with plasmids containing human growth hormone genomic sequences. The resultant variability in the production of antibody production was hypothesized to arise

from the operation of the microprojectile device, or the coating of the DNA onto the microprojectiles.

Ulmer *et al.* (*Science 259*:1745-1749 (1993)) injected a plasmid carrying the gene for influenza A nucleoprotein into the quadriceps of mice. The mice produced nucleoprotein antibodies, indicating that the gene was expressed in murine cells. The mice also produced nucleoprotein-specific cytotoxic T lymphocytes which were effective in protecting the mice from a subsequent challenge with a heterologous strain of influenza A virus. Similarly, Wang *et al.* (*Proc. Natl. Acad. Sci. USA 90*:4156-4160 (1993)) observed that the intramuscular injection of a human immunodeficiency virus (HIV) type 1 envelope DNA construct in mice generated antigen-specific cellular and humoral immune responses. In addition, splenic lymphocytes derived from the inoculated mice demonstrated HIV-envelope-specific proliferative responses. Thus, direct inoculation of DNA coding for pathogenic antigens can provide an alternative to the use of viruses, proteins, or peptides. See also U.S. Patent Nos. 5,593,972, 5,589,466 and 5,580,859.

Liposomes have been used as carriers of genetic information in the transfection of tissue culture cells. A fundamental problem of liposome-mediated transfection with liposomes comprising neutral or anionic lipids is that such liposomes do not generally fuse with the target cell surface. Instead, the liposomes are taken up phagocytically, and the polynucleotides are subsequently subjected to the degradative enzymes of the lysosomal compartment (Straubinger *et al.*, *Methods Enzymol. 101*:512-527 (1983); Mannino *et al.*, *Biotechniques 6*:682-690 (1988)). Another problem with conventional liposome technology is that the aqueous space of typical liposomes may be too small to accommodate large macromolecules such as DNA or RNA. As a result, typical liposomes have a low capturing efficiency (Felgner, "Cationic Liposome-Mediated Transfection with Lipofectin™ Reagent," in *Gene Transfer and Expression Protocols Vol. 7*, Murray, E.J., Ed., Humana Press, New Jersey, pp. 81-89 (1991)).

Liposomes comprising cationic lipids interact spontaneously and rapidly with polyanions such as DNA and RNA, resulting in liposome/nucleic acid complexes that capture 100% of the polynucleotide (Felgner *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 84*:7413-7417 (1987); Felgner *et al.*, *Focus 11*:21-25 (1989)). Moreover, the polycationic complexes are taken up by the anionic surface of tissue culture cells with an efficiency that is about ten to one hundred times greater than negatively charged or neutral liposomes (Felgner, "Cationic Liposome-Mediated Transfection with Lipofectin™ Reagent," in *Gene Transfer and Expression Protocols Vol. 7*, Murray, E.J., Ed., Humana Press, New Jersey, pp. 81-89 (1991)). In addition, the polycationic complexes fuse with cell membranes, resulting in an intracellular delivery of polynucleotide that bypasses the degradative enzymes of the lysosomal compartment (Düzgünes *et al.*, *Biochemistry 28*:9179-9184 (1989); Felgner *et al.*, *Nature 337*:387-388 (1989)).

Various formulations of cationic lipids have been used to transfect cells *in vitro* (WO 91/17424; WO 91/16024; U.S. Patent No. 4,897,355; U.S. Patent No. 4,946,787; U.S. Patent No. 5,049,386; and U.S. Patent No. 5,208,036). Cationic lipids have also been used to introduce foreign polynucleotides into frog and rat cells *in vivo* (Holt *et al.*, *Neuron 4*:203-214 (1990); Hazinski *et al.*, *Am. J. Respr. Cell. Mol. Biol. 4*:206-209 (1991)). Therefore, cationic lipids may be used, generally, as pharmaceutical carriers to provide biologically active substances (for example, *see* WO 91/17424; WO 91/16024; and WO 93/03709).

Direct gene transfer into the respiratory system could potentially be useful for either therapeutic or immunization purposes. There are several potential therapeutic applications for gene delivery at respiratory surfaces: (i) delivery of the cystic fibrosis (CF) transmembrane conductance regulator gene may be used to treat CF (Alton, E.W.F.W., *et al.*, *Nature Gen. 5*:135-142 (1993); Caplen, N.J., *et al.*, *Nature Med. 1*:39-46 (1995)); Yoshimura, K., *et al.*, *Nucleic Acids Res. 20*:3233-3240 (1992); (ii) transfer of the gene for gamma interferon (IFN-γ)

suppresses allergy-induced asthma by shifting a T-helper type 2 (Th2) immune response to a Th1 response (Li, X.-M., *et al.*, *J. Immunol. 157*:3216-3219 (1996)); (iii) lung cancer may be treated by direct transfer of suicide genes or tumor repressor genes (Wall, P.J., and Hart, I.R., *Annals Oncol. 6*:S73-S77 (1995); and (iv) genes encoding cytokines or coactivation factors may also be delivered for short-term, low dose systemic therapy.

Summary of the Invention

The present invention is directed to a method for transfecting mucosal epithelial cells of a mammal with a polynucleotide molecule, comprising:

(a) mixing at least one cationic lipid with a polynucleotide molecule, wherein the amount of lipid is sufficient to complex substantially said DNA molecule, thereby forming a cationic lipid-polynucleotide complex; and

(b) contacting the lipid-polynucleotide complex with the mucosal epithelia of said mammal, whereby said cells are transfected.

The polynucleotide molecule may be effective for gene therapy or code for an antigen capable of raising an immune response in the mammal.

The present invention is also directed to a method for generating an immune response against an infectious disease in an animal, comprising the steps of:

(a) mixing at least one cationic lipid with a polynucleotide coding for an antigen capable of raising an immune response in said mammal, wherein the amount of lipid is sufficient to complex substantially said DNA molecule, thereby forming a cationic lipid-polynucleotide complex; and

(b) administering the lipid-polynucleotide complex to the mucosal epithelia of the mammal;

whereby an immune response to the infectious disease is generated.

Complexes of the invention delivered to the epithelium are not only easy to administer, but also induce specific mucosal immunity, which is desirable to prevent entry of pathogens via the respiratory system or any of the other extensive mucosal surfaces of the body.

The present invention is further directed to a method for producing polyclonal antibodies comprising the use of the method of inducing an immune response described above. After production of the anitbodies, the invention may further comprise isolating the polyclonal antibodies from the immunized animal.

The present invention is also directed to a method for producing monoclonal antibodies, comprising:

(a) mixing at least one cationic lipid with a polynucleotide coding for an antigen capable of raising an immune response in a mouse, wherein the amount of lipid is sufficient to complex substantially said DNA molecule, thereby forming a cationic lipid-polynucleotide complex;

(b) administering the lipid-polynucleotide complex to at least one mammal;

(c) detecting whether an immune response to said antigen has occurred;

(d) removing B-lymphocytes from the mammal;

(e) fusing the B-lymphocytes with myeloma cells, thereby producing hybridomas;

(f) cloning the hybridomas;

(g) selecting positive clones which produce anti-immunogen antibody;

(h) culturing the anti-immunogen antibody-producing clones; and

(i) isolating anti-immunogen antibodies from the cultures.

The invention also relates to a cationic lipid mixture selected from the group consisting of:

(a) TMTPS:DOPE (preferably at a ratio of 1:1.5);

(b) TMTPS:C-16dl-PE (preferably at a ratio of 1:1.5);

(c) DMRIE:cholesterol (preferably at a ratio of 1:1);

(d) TMTOS:DOPE (preferably at a ratio of 1:1.5); and

(e) TMTOS:C-16dl-PE (preferably at a ratio of 1:1.5).

The invention also relates to a complex between a polynucleotide molecule and a cationic lipid mixture selected from the group consisting of:

(a) TMTPS:DOPE (preferably at a ratio of 1:1.5);

(b) TMTPS:C-16dl-PE (preferably at a ratio of 1:1.5);

(c) DMRIE:cholesterol (preferably at a ratio of 1:1);

(d) TMTOS:DOPE (preferably at a ratio of 1:1.5); and

(e) TMTOS:C-16dl-PE (preferably at a ratio of 1:1.5).

The invention also relates to a method of obtaining a polynucleotide/cationic lipid complex, comprising admixing a polynucleotide with at least one cationic lipid in an amount sufficient to complex substantially said polynucleotide molecule, thereby forming a cationic lipid-polynucleotide complex.

The invention also relates to a polynucleotide/cationic lipid complex obtained by the method of the invention.

Brief Description of the Figures

FIG. 1. depicts a bar graph showing luciferase reporter gene activity in mouse lung tissue 48 hr after IN inhalation of 25 μg of pCMV-luc DNA associated with lipid 301 at various DNA-lipid (w/w) ratios. Each bar indicates mean total luciferase activity in relative light units per second (RLU/sec) (n=10), and T bars indicate the standard errors of the mean.

FIG. 2. depicts a bar graph showing luciferase reporter gene activity in mouse lung tissue 48 hr after IN inhalation of various doses of pCMV-luc DNA

associated with lipid 301 at a 1:1 DNA:lipid (w/w) ratio. Each bar indicates mean total luciferase activity in relative light units per second (RLU/sec) (n=10), and T bars indicate the standard errors of the mean.

FIG. 3. depicts a bar graph showing luciferase reporter gene activity in mouse lung tissue at various times after IN inhalation of 100 μg of pCMV-luc DNA associated with lipid 301 at 1:1 DNA:lipid (w/w) ratio. Each bar indicates mean total luciferase activity in relative light units per second (RLU/sec) (n=10), and T bars indicate the standard errors of the mean.

FIG. 4. depicts a graph showing luciferase reporter gene activity in mouse lung tissue at 24 (triangles), 48 (circles) or 72 (squares) hours after administration of 100 μg of pCMV-luc DNA in saline (open symbols) or associated with lipid 301 at a 1:1 DNA:lipid (w/w) ratio (closed symbols). DNA solutions were administered by intranasal (IN) instillation or inhalation, or by intratracheal (IT) injection or cannulation. Each point represents the value obtained from a single animal.

FIG. 5. depicts a bar graph showing luciferase reporter gene activity in mouse lung tissue 48 hr after IN inhalation of 100 μg of pCMV-luc DNA in saline or associated with one of 14 different lipids at 1:1 DNA:lipid (w/w) ratio. Each bar indicates mean total luciferase activity in relative light units per second (RLU/sec) (n=10), and T bars indicate the standard errors of the mean.

FIGs. 6A-6C depict the structures of preferred cationic lipids for use in the present invention.

FIG. 7 depicts the general procedure for the synthesis of C12, C14, C16 and C18 tetramethyl tetraalkyl spermine analogs.

Detailed Description of the Preferred Embodiments

Definitions

In the description that follows, a number of terms are utilized extensively. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Cloning vector. A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, provide tetracycline resistance or ampicillin resistance.

Expression vector. A vector similar to a cloning vector but which is capable of enhancing the expression of a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Promoter sequences may be either constitutive or inducible.

Recombinant Host. In general, a recombinant host may be any prokaryotic or eukaryotic microorganism or cell which contains the desired cloned genes on an expression vector or cloning vector. This term is also meant to include those microorganisms that have been genetically engineered to contain the desired gene(s) in the chromosome or genome of that organism.

Recombinant vector. Any cloning vector or expression vector which contains the desired cloned gene(s).

Host. Any prokaryotic or eukaryotic microorganism or cell that is the recipient of a replicable expression vector or cloning vector. A "host," as the term is used herein, also includes prokaryotic or eukaryotic microorganisms or cells that can be genetically engineered by well known techniques to contain desired gene(s) on its chromosome or genome. For examples of such hosts, *see* Maniatis *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982).

Promoter. A DNA sequence generally described as the 5′ region of a gene, located proximal to the start codon. The transcription of an adjacent gene(s) is initiated at the promoter region. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter. Examples of promotors that may be used to drive gene expression in epithelial mucosal cells *in vivo* include, but are not limited to the CMV promoter (InVitrogen, San Diego, CA), the SV40, MMTV, and hMTIIa promoters (U.S. 5,457,034), the HSV-1 4/5 promoter (U.S. 5,501,979), and the early intermediate HCMV promoter (WO92/17581).

Gene. A DNA sequence that contains information needed for expressing a polypeptide or protein.

Structural gene. A DNA sequence that is transcribed into messenger RNA (mRNA) that is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Therapeutic Gene. A gene that when transfected and expressed *in vivo* into mammalian host cells results in the cure, amelioration or prevention of a mammalian disease.

Antisense oligonucleotide. A DNA or RNA molecule or a derivative of a DNA or RNA molecule containing a nucleotide sequence which is complementary to that of a specific mRNA. An antisense oligonucleotide binds to the complementary sequence in a specific mRNA and inhibits translation of the mRNA. There are many known derivatives of such DNA and RNA molecules.

See, for example, U.S. Patent Nos. 5,602,240, 5,596,091, 5,506,212, 5,521,302, 5,541,307, 5,510,476, 5,514,787, 5,543,507, 5,512,438, 5,510,239, 5,514,577, 5,519,134, 5,554,746, 5,276,019, 5,286,717, 5,264,423, as well as WO96/35706, WO96/32474, WO96/29337 (thiono triester modified antisense oligodeoxynucleotide phosphorothioates), WO94/17093 (oligonucleotide alkylphosphonates and alkylphosphothioates), WO94/08004 (oligonucleotide phosphothioates, methyl phosphates, phosphoramidates, dithioates, bridged phosphorothioates, bridge phosphoramidates, sulfones, sulfates, ketos, phosphate esters and phosphorobutylamines (van der Krol *et al., Biotech. 6*:958-976 (1988); Uhlmann *et al., Chem. Rev. 90*:542-585 (1990)), WO94/02499 (oligonucleotide alkylphosphonothioates and arylphosphonothioates), and WO92/20697 (3'-end capped oligonucleotides). *See also*, Jack Cohen, *Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression*, CRC Press (1989)). S-oligos (nucleoside phosphorothioates) are isoelectronic analogs of an oligonucleotide (O-oligo) in which a nonbridging oxygen atom of the phosphate group is replaced by a sulfur atom. The S-oligos of the present invention may be prepared by treatment of the corresponding O-oligos with 3*H*-1,2-benzodithiol-3-one-1,1-dioxide which is a sulfur transfer reagent. *See* Iyer *et al., J. Org. Chem. 55*:4693-4698 (1990); and Iyer *et al., J. Am. Chem. Soc. 112*:1253-1254 (1990).

Antisense Therapy. A method of treatment wherein antisense oligonucleotides are administered to a patient in order to inhibit the expression of the corresponding protein.

Expression. Expression is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

Transfection. Transfection refers to the stable of transient transformation of a host cell, e.g. a mucosal epithelial cell, with a nucleic acid molecule, e.g. a DNA molecule. The recombinant host cell expresses protein which is encoded by the transfected DNA.

Antigenic Determinant. A protein or peptide which contains one or more epitopes.

Immunogen. A protein or peptide which is capable of eliciting an immune response due to the presence of one or more epitopes.

The present invention is directed to a method for transfecting mucosal epithelial cells of a mammal with a polynucleotide molecule, comprising:

(a) mixing at least one cationic lipid with a polynucleotide molecule, wherein the amount of lipid is sufficient to complex substantially said DNA molecule, thereby forming a cationic lipid-polynucleotide complex; and

(b) administering the lipid-polynucleotide complex to the mucosal epithelia of said mammal.

As described herein, this method may be used to generate an immune response in a mammal or to practice gene therapy, e.g. with therapeutical effective genes, antisense oligonucleotides, ribozymes, external guide sequences and the like.

I. *Cationic Lipids*

Any of the cationic lipids known in the prior art may be employed in the practice of the claimed invention. *See*, for example, Felgner *et al.* (*Proc. Natl. Acad. Sci. U.S.A. 84*:7413-7417 (1987)); Felgner *et al.* (*Focus 11*:21-25 (1989)); Felgner ("Cationic Liposome-Mediated Transfection with Lipofectin™ Reagent," in *Gene Transfer and Expression Protocols Vol. 7*, Murray, E.J., Ed., Humana Press, New Jersey, pp. 81-89 (1991)); WO 91/17424; WO 91/16024; U.S. Patent Nos. 4,897,355, 4,946,787, 5,049,386, 5,208,036, 5,580,859, 5,589466 and 5,593,972; Behr *et al.* (*Proc. Natl. Acad. Sci. USA 86*:6982-6986 (1989); EPO Publication 0 394 111); Gao *et al.* (*Biochim. Biophys. Res. Comm. 179*:280-285 (1991)); Zhou *et al.*, (*Biochim. Biophys.*

Res. Comm. 165:8-14 (1991)); and Gebeyehu *et al.* (co-owned U.S. application serial no. 07/937,508; filed August 28, 1992).

Example structures of cationic lipids useful in this invention are provided in Table 1. Generally, any cationic lipid, either monovalent or polyvalent, can be used in the compositions and methods of this invention. Polyvalent cationic lipids are generally preferred. Cationic lipids include saturated and unsaturated alkyl and alicyclic ethers and esters of amines, amides or derivatives thereof. Straight-chain and branched alkyl and alkene groups of cationic lipids can contain from 1 to about 25 carbon atoms. Preferred straight-chain or branched alkyl or alkene groups have six or more carbon atoms. Alicyclic groups can contain from about 6 to 30 carbon atoms. Preferred alicyclic groups include cholesterol and other steroid groups. Cationic lipids can be prepared with a variety of counter ions (anions) including among others: Cl^-, Br^-, I^-, F^-, acetate, trifluoroacetate, sulfate, nitrite, and nitrate.

A well-known cationic lipid is N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). See Felgner, P.L. *et al. Proc. Natl. Acad. Sci. USA 84*:7413-7417 (1987). DOTMA and the analogous diester DOTAP (1,2-bis(oleoyloxy)-3-3-(trimethylammonium)propane), see Table 1 for structures, are commercially available. Additional cationic lipids structurally related to DOTMA are described in U.S. Patent No. 4,897,355.

Another useful group of cationic lipids related to DOTMA and DOTAP are commonly called DORI-ethers or DORI-esters. DORI lipids differ from DOTMA and DOTAP in that one of the methyl groups of the trimethylammonium group is replaced with a hydroxyethyl group, see structure in Table 1. The DORI lipids are similar to the Rosenthal Inhibitor (RI) of phospholipase A (Rosenthal, A.F. and Geyer, R.P., *J. Biol. Chem. 235*:2202-2206 (1960). The oleoyl groups of DORI lipids can be replaced with other alkyl or alkene groups such as palmitoyl or stearoyl groups. The hydroxyl group of the DORI-type lipids can be used as a site for further functionalization, for example, for esterification to amines, like carboxyspermine.

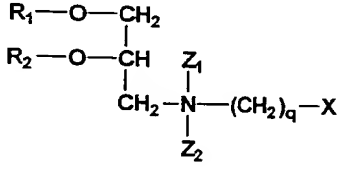
Additional cationic lipids which can be employed in the compositions and methods of this invention include those described as useful for transfection of cells in PCT application WO 91/15501 published Oct. 17, 1991, Pinnaduwage, P. *et al., Biochem. Biophys. Acta. 985*:33-37 (1989); Rose, J.K., *et al., BioTechniques 10*:520-525 (1991), Ho, A. *et al., Biochem. Intern. 22*:235-241 (1990).

Cationic sterol derivatives, like 3β[N-(N',N'-dimethyl-aminoethane)carbamoyl] cholesterol (DC-Chol) in which cholesterol is linked to a trialkylammonium group, see Table 1, can also be employed in the present invention. DC-Chol is reported to provide more efficient transfection and lower toxicity than DOTMA-containing liposomes for some cell lines. (Goa, X. and Huang, L., *Biochem. Biophys. Res. Comm. 179*:280-285 (1991).

The polycationic lipid formed by conjugating polylysine to DOPE (Zhou, X. *et al., Biochem. Biophys.* Acta 1065:8-14 (1991)), as well as other lipopolylysines, can also be employed in this invention.

Polycationic lipids containing carboxyspermine are also useful in the practice of this invention. Behr, J-P. *et al., Proc. Natl. Acad Sci. (USA) 82*:6982-6986 (1989), and EPO published application 304 111 (1990) describe carboxyspermine-containing cationic lipids including 5-carboxyspermylglycine dioctadecyl-amide (DOGS) and dipalmitoyl-phosphatidylethanolamide 5-carboxyspermylamide (DPPES). Additional cationic lipids can be obtained by replacing the octadecyl and palmitoyl groups of DOGS and DPPES, respectively, with other alkyl or alkene groups.

U.S. Pat. No. 5,334,761 describes cationic lipids of Formula Ia which are useful in this invention:



Ia

where R_1 and R_2 separately or together are C_{1-23} alkyl or alkenyl or ($-CO-C_{1-23}$) alkyl or alkenyl, q is 1 to 6, Z_1 and Z_2, separately or together, are H or an unbranched alkyl group having one to six carbon atoms and where X can be a variety of groups including haloalkyl, alkylamines, alkyldiamines, alkyltriamines, alkyltetraamines, carboxyspermine and related amines, or polyamines including polylysine or polyarginine.

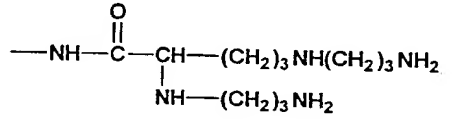
Compounds of Formula Ia in which X is a nitrogen-containing group such as:

$-(CH_2)_nNH_2$, where n = 0-6 (X1);

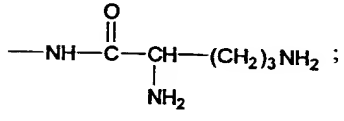
$-NH-(CH_2)_m-NH_2$, wherein m = 2-6 (X2);

$-NH-(CH_2)_3-NH-(CH_2)_4NH_2$ (X3);

$-NH-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$ (X4);

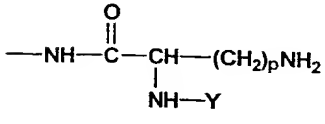


(X5)



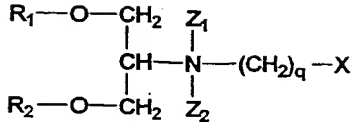
(X6)

or



where p is 2-5 and Y is H or a group attached by an amide or alkyl amino group (X7) are particularly useful for complexation to nucleic acids. Polycationic lipids, such as those of Formula Ia where X is a spermine (e.g. X5) are preferred.

Altenatively, the compounds of Formula Ib may also be employed:



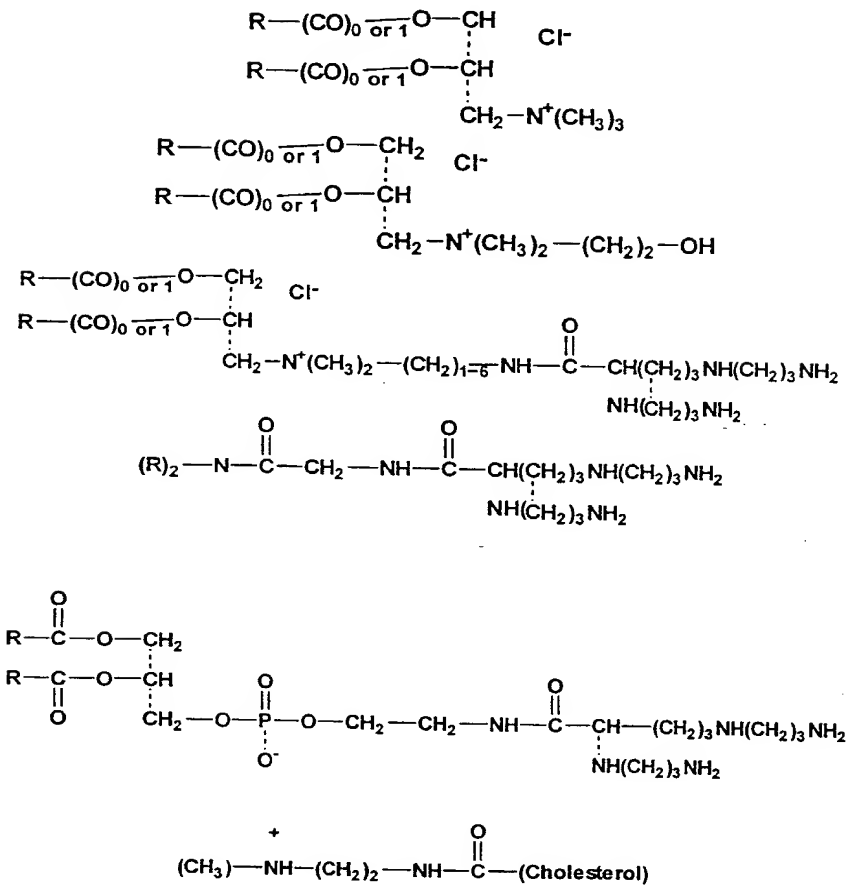
Ib

wherein R_1, R_2, Z_1, Z_2, q and X are defined above with respect to Formula Ia.

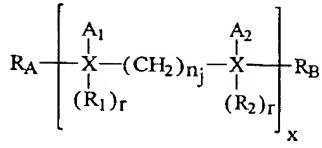
In the transfection compositions employed in this invention, cationic lipids can optionally be combined with non-cationic lipids, preferably neutral lipids, to form lipid aggregates that complex with nucleic acids. Neutral lipids useful in this invention include, among many others: lecithins; phosphotidylethanolamine; phosphatidyllethanolamines, such as DOPE (dioleoylphosphatidylethanolamine), POPE (palmitoyloleoylphosphatidylethanolamine) and distearoylphosphatidylethanolamine; phosphotidylcholine; phosphatidylcholines, such as DOPC (dioleoylphosphotidylcholine), DPPC (dipalmitoylphosphatidylcholine), POPC (palmitoyloleoylphosphatidylcholine) and distearoylphosphatidylcholine; phosphatidylglycerol; phosphatidylglycerols, such as DOPG (dioleoylphosphatidylglycerol), DPPG (dipalmitoylphosphatidylglycerol), and distearoylphosphatidylglycerol; dipalmitoyl phosphatidyl ethanolamine (C-16-PE, FIG. 6A); dipalmitoleoyl phosphatidyl ethanolamine (C-16dl-PE, FIG. 6A); phosphatidylserine; phosphatidylserines, such as dioleoyl- or dipalmitoylphosphatidylserine; diphosphatidylglycerols; fatty acid esters; glycerol esters; sphingolipids; cardolipin; cerebrosides; and ceramides; and mixtures thereof. Neutral lipids also include cholesterol and other 3βOH-sterols.

Table 1:

Examples of Cationic Lipids



Also useful in the practice of the present invention are highly packed polycationic ammonium, sulfonium and phosphonium lipid compounds described in WO95/17373 (and U.S. appln. no. 08/171,232) according to the general Formula (II):



In the general Formula (II),

X is selected from the group consisting of N, S, P or SO;

x is an integer ranging from 1 to about 20;

n_j, where j = 1 to x, are, independently of one another, integers that can have a value ranging from 1 to about 6;

R_A and R_B, independently of one another, are selected from the group consisting of H, or an alkyl, hydroxyalkyl or thiol substituted alkyl group having from 1 to about 6 carbon atoms;

R_1 and R_2, independently of one another, are selected from the group consisting of alkyl groups having from 1 to about 6 carbon atoms, where r is either 1 or 0, such that r is 0 or 1 when X is N, r is 0 when X is S or SO, and r is 1 when X is P; and

A_1-A_2, independently of one another, are selected from the group consisting of the following groups Z_1-Z_6:

Z_1 is a straight-chain alkyl, alkenyl, or alkynyl group having from 2 to about 22 carbon atoms wherein one or more non-neighboring —CH_2— groups can be replaced with an 0 or S atom;

Z_2 is a branched alkyl, alkenyl, or alkynyl group having from 2 to about 22 carbon atoms wherein one or more non-neighboring -CH_2- groups can be replaced with an 0 or S atom;

Z_3 is a straight-chain or branched alkyl group substituted with one or two OH, SH, NH_2 or amine groups within about 3 carbon atoms of the bond between Z_3 and X;

Z_4 is a substituted straight-chain or branched alkyl, alkenyl or alkynyl group having from 2 to about 22 carbon atoms wherein the substituent is an

aromatic, alicyclic, heterocyclic or polycyclic ring and wherein one or more of the non-neighboring $-CH_2-$ groups of said alkyl, alkenyl or alkynyl group can be substituted with an 0 or S atom.

Z_5 is a -B-L group wherein B is selected from the group $-CO-$, $-CO_2-$, $-OCO-$, $-CO-N-$, $-O-CO-N-$, $-O-CH_2-$, $-CH_2-O-$, $-S-$, CH_2-, $-CH_2-S-$ or $-CH_2-$ and L is selected from the group consisting of:

Z_1; Z_2; Z_4; or

an aromatic, alicyclic, heterocyclic or polycyclic ring moiety;

Z_6 is a $-CH(D-L)_2$ or a $-C(D-L)_3$ group wherein D is selected from the group consisting of $-CO-$, $-CO_2-$, $-OCO-$, $-CO-N-$, $-O-CO-N-$, $-O-$, or $-S-$ and L is selected from the group consisting of:

Z_1; Z_2; Z_4; or

an aromatic, alicyclic, heterocyclic or polycyclic ring moiety.

In general in any particular compound of Formula II, the chain length n_j can vary from 1 to 6. For example, in a compound in which x is 3 where j is 1 to 3, n_1, n_2 and n_3 can all have the same value, any two can have the same value or all three can have different values. In particular embodiments, the chain length n_j vary in the repeating pattern 3, 4, 3, 3, 4, 3, ... other particular embodiments of this invention include those in which n_j differ from each other by +/- 1.

A groups, A_1-A_4 include those in which two substituents on different X's, preferably neighboring X groups, are covalently linked with each other to form a cyclic moiety.

The oxygen or sulfur atoms introduced into Z_1, and Z_2 groups are preferably introduced within about 3 carbon atoms from the bond to the X group.

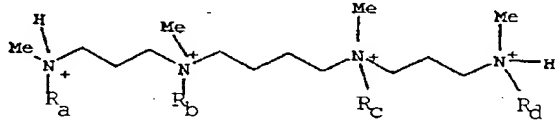
The aromatic, alicyclic, heterocyclic or polycyclic ring moieties can be substituted or unsubstituted. Substituents include among others: OH, SH, NH_2, CH_3, $COCH_3$ and halogens, particularly F. Further, one or more of the ring carbons in the alicyclic, heterocyclic or polycyclic ring moieties of this invention can be carbonyl groups C=O.

Introduction of OH, NH_2 or amine groups as substituents on A groups within about 3 carbon from the bond to X can facilitate solubility of the compounds in physiological media.

The cationic lipids of Formula II are useful, either alone or in combination with other neutral lipid aggregate-forming components (e.g., DOPE or cholesterol) for formulation into liposomes or other lipid aggregates. Such aggregates are polycationic, able to form stable complexes with anionic macromolecules, such as nucleic acids. The polyanion-lipid complex interacts with cells making the polyanionic macromolecule available for absorption and uptake by the cell.

Of special interest are the products of general Formula (II) in which X is nitrogen and A_1 and A_2 are Z_1. These N-alkylated polyamines and their quaternary ammonium salts are particularly useful for intracellular delivery of negatively charged macromolecules.

Also useful in the practice of the present invention are lipids having Formula III:



III

wherein the groups R_a, R_b, R_c and R_d are C12, C13, C14, C15, C16, C17, C18, C19, C20, C21 or C22 straight chain alkyl or alkenyl groups. See, for example, FIG. 6C (compound XXVII) and FIG. 7. In a prefered embodiment, the longer chain lipids (C18-C22) lipids are employed.

Other preferred lipids include those in the following Table 2:

Table 2

Code #	Name (abreviation) (molar ratio (about))	Structure
301	CellFectin (TMTPS:DOPE)(1:1.5)	TMTPS I DOPE II
302	TMTPS:C-16dl-PE (1:1.5)	TMTPS I C-16dl-PE IV
303	DMRIE-C (DMRIE:Cholesterol) (1:1)	DMRIE XXV
304	GAP-DLRIE:DOPE (1:1)	GAP-DLRIE XXVI
305	LipofectAmine (DOSPA:DOPE) (1.5:1)	
9231	TMTOS:DOPE (1:1.5)	TMTOS XXVII
9232	TMTOS:C-16dl-PE (1:1.5)	
201	TMTMS:DOPE (1:1.5)	TMTMS VII
202	TMTMS:C-16dl-PE (1:1.5)	
203	TMTMS:C-14dl-PE (1:1.5)	C-14dl-PE (same as C-16dl-PE but with 2 less CH_2's in the two chains)
204	TMTLS:DOPE (1:1.5)	TMTLS VI
205	TMTLS:C-16dl-PE (1:1.5)	
206	TMTLS:C-12-PE (1:1.5)	C-12-PE (similar as C-16dl-PE but with 4 less CH_2's in the two chains and no double bonds)

The structures for Formulae I, II, III, IV, VI, VII, VIII, XXV, XXVI and XXVII appear in FIGs. 6A-6C.

II. Genes Useful in the Present Invention.

Direct gene transfer to mammalian cells *in vivo* may be carried out with the use of polynucleotides, e.g. plasmid DNA, or by using a viral or bacterial vector delivery system. The use of plasmid DNA vectors is highly preferable to live attenuated vectors owing to the ease of production, non-immunogenic nature of the vector itself, and absence of risk of inadvertent infection. In addition, circular DNA is preferable to linear DNA.

Plasmid DNA may be used either in a pure form ("naked") or formulated with cationic lipids, however there are conflicting reports in the literature as to their relative efficiencies for directly transferring foreign genes into lung tissue. The present invention relates to studies undertaken to examine the efficiency of gene transfer with different techniques of DNA delivery to the lungs and compare the use of pure plasmid DNA and DNA formulated with 14 different lipids.

Particular therapeutic genes that can be used in the practice of the invention include, but are not limited to, the cystic fibrosis (CF) transmembrane conductance regulator gene used to treat CF (Alton, E.W.F.W., *et al., Nature Gen. 5*:135-142 (1993); Caplen, N.J., *et al., Nature Med. 1*:39-46 (1995); Yoshimura, K., *et al., Nucleic Acids Res. 20*:3233-3240 (1992); Riordan, J.R. *et al., Science 245*:1066 (1989)); the gene for gamma interferon (IFN-γ)(Li, X.-M., *et al., J. Immunol. 157*:3216-3219 (1996)); suicide genes or tumor repressor genes (Wall, P.J., and Hart, I.R., *Annals Oncol. 6*:S73-S77 (1995); genes encoding cytokines or coactivation factors; factor VIII (hemophilia A) (*see,* e.g. Wood *et al., Nature 312*:330 (1984)), factor IX (hemophilia B) (*see,* e.g. Kurachi, K. *et al., Proc. Natl. Acad. Sci USA 79*:6461 (1982)), adenosine deaminase (SCID) (*see,* e.g. Valerio, D. *et al., Gene 31*:147 (1984)), or α-1 antitrypsin (emphysema of the lungs) (*see,* e.g. Ciliberto, G. *et al., Cell 41*:531 (1985)).

Therapeutic application may also be *ex vivo*, in which the treated cells, e.g. epithelial cells, are returned to the body. (See, Ponder *et al.*, *Proc. Natl. Acad. Sci. USA 88*:1217-1221 (1991).)

Antisense oligonucleotides have been described as naturally occurring biological inhibitors of gene expression in both prokaryotes (Mizuno *et al.*, *Proc. Natl. Acad. Sci. USA 81*:1966-1970 (1984)) and eukaryotes (Heywood, *Nucleic Acids Res. 14*:6771-6772 (1986)), and these sequences presumably function by hybridizing to complementary mRNA sequences, resulting in hybridization arrest of translation (Paterson, *et al.*, *Proc. Natl. Acad. Sci. USA, 74*:4370-4374 (1987)).

Antisense oligonucleotides are short synthetic DNA or RNA nucleotide molecules formulated to be complementary to a specific gene or RNA message. Through the binding of these oligomers to a target DNA or mRNA sequence, transcription or translation of the gene can be selectively blocked and the disease process generated by that gene can be halted (*see*, for example, Jack Cohen, *Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression*, CRC Press (1989)). The cytoplasmic location of mRNA provides a target considered to be readily accessible to antisense oligodeoxynucleotides entering the cell; hence much of the work in the field has focused on RNA as a target. Currently, the use of antisense oligodeoxynucleotides provides a useful tool for exploring regulation of gene expression *in vitro* and in tissue culture (Rothenberg, *et al.*, *J. Natl. Cancer Inst. 81*:1539-1544 (1989)).

Antisense therapy is the administration of exogenous oligonucleotides which bind to a target polynucleotide located within the cells. For example, antisense oligonucleotides may be administered for anticancer therapy (U.S. Patent No. 5,271,941).

Ribozymes provide an alternative method to inhibit mRNA function. Ribozymes may be RNA enzymes, self-splicing RNAs, and self-cleaving RNAs (Cech *et al.*, *Journal of Biological Chemistry 267*:17479-17482 (1992)). It is possible to construct *de novo* ribozymes which have an endonuclease activity

directed in *trans* to a certain target sequence. Since these ribozymes can act on various sequences, ribozymes can be designed for virtually any RNA substrate. Thus, ribozymes are very flexible tools for inhibiting the expression of specific genes and provide an alternative to antisense constructs.

A ribozyme against chloramphenicol acetyltransferase mRNA has been successfully constructed (Haseloff *et al.*, *Nature 334*:585-591 (1988); Uhlenbeck *et al.*, *Nature 328*:596-600 (1987)). The ribozyme contains three structural domains: 1) a highly conserved region of nucleotides which flank the cleavage site in the 5' direction; 2) the highly conserved sequences contained in naturally occurring cleavage domains of ribozymes, forming a base-paired stem; and 3) the regions which flank the cleavage site on both sides and ensure the exact arrangement of the ribozyme in relation to the cleavage site and the cohesion of the substrate and enzyme. RNA enzymes constructed according to this model have already proved suitable *in vitro* for the specific cleaving of RNA sequences (Haseloff *et al.*, *supra*).

Alternatively, hairpin ribozymes may be used in which the active site is derived from the minus strand of the satellite RNA of tobacco ring spot virus (Hampel *et al.*, *Biochemistry 28*:4929-4933 (1989)). Recently, a hairpin ribozyme was designed which cleaves human immunodeficiency virus type 1 RNA (Ojwang *et al.*, *Proc. Natl. Acad. Sci. USA 89*:10802-10806 (1992)). Other self-cleaving RNA activities are associated with hepatitis delta virus (Kuo *et al.*, *J. Virol. 62*:4429-4444 (1988)). See also U.S. 5,574,143 for methods of preparing and using ribozymes. Alternatively, ribozyme molecules are designed as described by Eckstein *et al.* (International Publication No. WO 92/07065) who disclose catalytically active ribozyme constructions which have increased stability against chemical and enzymatic degradation, and thus are useful as therapeutic agents.

In an alternative approach, an external guide sequence (EGS) can be constructed for directing the endogenous ribozyme, RNase P, to intracellular mRNA, which is subsequently cleaved by the cellular ribozyme (Altman *et al.*,

U.S. Patent No. 5,168,053). Preferably, the EGS comprises a ten to fifteen nucleotide sequence complementary to the target mRNA (corresponding to the miss-sequenced regions) and a 3'-NCCA nucleotide sequence, wherein N is preferably a purine (*Id.*). After EGS molecules are delivered to cells, the molecules bind to the targeted mRNA species by forming base pairs between the mRNA and the complementary NTP EGS sequences, thus promoting cleavage of mRNA by RNase P at the nucleotide at the 5'side of the base-paired region (*Id.*).

Exemplary genes coding for antigenic proteins capable of inducing an immune response when transfected into mucosal epithelial cells include outer membrane proteins from *N. meningitidis* (U.S. 5,439,808), outer membrane proteins from *H. influenzae* (U.S. 5,196,338, EP 320,289, EP 378,929, WO95/03069, Munson and Tolan, *Infect. Immun. 57*:88 (1989)), pneumococcal proteins (U.S. 5,476,929); pseudorabies virus proteins (U.S. 4,753,884, U.S. 4,609,548), measles virus proteins (U.S. 5,503,834), herpesvirus proteins (U.S. 5,482,713, U.S. 4,824,667, U.S. 4,859,587, U.S. 5,324,664, U.S. 5,462,734, U.S. 5,266,489, U.S. 5,529,780), *Vibio cholerae* proteins (U.S. 5,066,596), poxvirus proteins (U.S. 5,514,375, U.S. 5,368,855, 5,266,313, U.S. 5,453,364, U.S. 5,338,679, 5,204,243), hepatitis B viral proteins (U.S. 5,314,808, U.S. 5,196,194); papilloma virus capsid proteins (U.S. 5,437951), *Borrelia burgdorferi* proteins (U.S. 5,582,990, U.S. 5,523,089, U.S. 5,246,844), mycobacterial proteins (U.S. 5,559,011, U.S. 5,026,636, U.S. 5,330,754); *Pasteurella harmolytica* glycoprotease (U.S. 5,543,312), and *Helicobacter pylori* proteins (U.S. 5,527,678).

III. Preparation of the Lipid/Polynucleotide Complexes

Preferably, liposomes comprising the lipids are first prepared by the reverse evaporation method.

Cationic lipid-polynucleotide complexes are formed by mixing a cationic lipid solution, e.g. containing liposomes, with a polynucleotide solution. Preferably, the complexes are mixed by vortexing. The cationic lipid and polynucleotides can be dissolved in any sterile physiologically-compatible aqueous carrier. Preferably, cationic lipid and polynucleotides are dissolved in sterile saline (150 mM NaCl). The solutions are mixed at ambient temperatures. Preferably, the solutions are mixed at 25 °C. After mixing, the cationic lipid-polynucleotide complexes are incubated at room temperature, preferably for 15 to 45 minutes.

It has been discovered that the ratio of lipid to DNA is important to the transfection efficiency of the complex. In general, one must combine an amount of lipid that is sufficient to complex substantially the polynucleotide molecule. This amount of lipid can be determined empirically titrating the polynucleotide molecule with the lipid and separating the reaction mixture on an agarose gel. The presence of uncomplexed polynucleotide molecule indicates that more lipid should be added. Depending on the particular lipid or lipid mixture being employed, the ratio of polynucleotide to lipid may range from about 5:1 to 1:50 (w/w), more preferably, about 2:1 ot 1:5 or 1:10, even more preferably about 1:1 to 1:3, and even more preferably about 1:2. The most prefered ratio of polynucleotide to lipid is where the DNA is fully complexed without substantial amounts of excess lipid as determined on a gel. An excess of lipid is to be avoided due to possible toxicity of the lipid.

For gene delivery, plasmid DNA may be formulated with cationic liposomes, which are themselves composed of a mixture of a cationic lipid and a neutral co-lipid. The electrostatic interactions between the net-positively charged lipid and a negatively charged DNA result in the spontaneous formation of lipid-DNA complexes. The neutral lipid acts to increase the stability of the liposome, reduce cytotoxity and improve transfection efficiency.

IV. Use of the Lipid/Polynucleotide Complexes

According to the present invention, the lipid/polynucleotide complex is used to carry out an *in vivo* transfection of mammalian mucosal epithelial cells. Examples of mucosal epithelial cells that may be transfected include those cells in the nasal passageways, the lung, inside the cheek, under the tongue, the rectum, intestines and vagina. Administration of lipid/polynucleotide complexes of the present invention may be by any suitable means that will result in administration to target mucosal epithelium. Such means include intranasal administration, instillation, inhalation, injection and cannulation. Most preferably, the lipid complexes are administered as part of a nasal spray, or inhaled via nebulization into the mouth and/or nose, or via an endotracheal tube.

The methods of the invention may be practiced on any mammal which may experience the beneficial effects of the invention. Preferably, such mammals are humans, although the invention is not intended to be so limiting. Other examples of mammals that may be treated according to the invention include sheep, goats, cattle, horses, dogs, cats and pigs.

Transfected cells express a foreign protein encoded by the polynucleotide, and may present the foreign protein on the cell surface. As a result, the host animal mounts an immune response to the foreign protein, or immunogen. Thus, the lipid/polynucleotide complex can be used as a vaccine to induce an immune response in a mammal.

The vaccines comprising the cationic lipid and polynucleotide may be administered in a wide range of dosages. Effective dosages and formulations will depend upon a variety of factors (such as the species of the recipient), and can be determined by one of ordinary skill in the art. Illustrative dosages range from about 1 μg to about 500 μg, depending on the animal. For example, the dose for a mouse may be only 10-50% of the human dose. A human baby will

require a dose of about 50% of the adult dose. Such doses may be optimized by the clinician with no more than routine experimentation.

When administered for the purposes of gene therapy, higher dosages of the therapeutic gene are required. Illustrative dosages range from about 100 μg to about 5 mg.

The specific dosage administered may be dependent upon the age, weight, kind of current treatment, if any, and nature of the gene which will be expressed. In the cases of genes coding for immunogens, the initial dose may be followed by a booster dosage after a period of about four weeks to enhance the immunogenic response.

As described above, genetic immunization protocols in which naked DNA is administered can require as much as 500 μg or more of a DNA construct per inoculation. In contrast, the use of particular cationic lipids as a carrier for DNA constructs according to the claimed invention permits genetic immunization with much lower amounts of a DNA construct. Use of lower amounts of DNA constructs is important when the construct is not available in large quantities.

The lipid/polynucleotide complexes may be administered to the epithelium of the mammal as part of a pharmaceutical composition comprising a pharmaceutically acceptable carrier such as buffered physiologic saline solution.

V. The Preparation of Antibodies

Since immunization generates the production of immunogen-specific antibodies in the vaccine, the present invention is also directed to methods of producing immunogen-specific antibodies. Polyclonal antibodies may be isolated and purified from vaccinated animals using procedures well-known in the art (for example, *see* Harlow *et al., Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1988).

This invention is also directed to the use of immunization to produce monoclonal antibodies. According to this method, a mammal (e.g. a mouse) is injected with a lipid/polynucleotide complex, and after the mammal has generated an immune response, B-lymphocytes are isolated from the mammal. The immune response may be monitored by checking the antibody titer of the sera. Monoclonal antibodies are produced following the procedure of Köhler and Milstein (*Nature* 256:495-497 (1975) (for example, *see* Harlow *et al., supra*). Briefly, monoclonal antibodies can be produced by immunizing mammals with a cationic lipid-polynucleotide complex, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce anti-immunogen antibody, culturing the anti-immunogen antibody-producing clones, and isolating anti-immunogen antibodies from the hybridoma cultures.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

Examples

Example 1 Synthesis of C12, C14, C16 and C18 Tetramethyl Tetraalkyl Spermine Analogs

The general procedure for the preparation of these C12, C14, C16 and C18 tetramethyl tetraalkyl spermine analogs is depicted in **FIG. 7**.

In a 500 mL round bottom flask, spermine (8.31 mmole) and triethyl amine (41.5 mmole) were dissolved in 90 mL of dry dichloromethane (distilled after refluxing over CaH_2 for two hours) under argon atmosphere. The reaction mixture was cooled to 0°C with an ice bath. A solution of fatty acid chloride (33.2 mmole) in 30 mL of dry dichloromethane was transferred slowly to the

reaction mixture under stirring over a period of 20 minutes. The reaction mixture was warmed to room temperature and stirred in the dark under argon atmosphere for an additional 48 hours. The mixture was quenched with water at 0°C and mixed with 350 mL of chloroform. The aqueous layer was separated and extracted twice with 300 mL of 5% $NaHCO_3$, dried over anhydrous Na_2SO_4 for 40 minutes and concentrated. The crude product was dried under a vacuum overnight and analyzed by IR and NMR as the tetraacyl spermine. It was used in the next step without further purification.

To a suspension of the tetraacyl spermine (8.3 mmole) in 200 mL of anhydrous Et_2O was added a solution of LAH (41.5 mmole) slowly at 0°C. The reaction mixture was further diluted with 100 mL of anhydrous Et_2O. The reaction mixture was slowly warmed to room temperature for another 30 minutes then heated to reflux with a condenser. After 40 hours of refluxing, the mixture was cooled and quenched at 0°C very carefully with 30 mL of 0.5 N NaOH solution. The mixture was stirred at room temperature for an additional 4 hours. The organic layer was then decanted. The aqueous layer was washed twice with 100 mL of THF. The combined organic layer was washed with saturated NaCl solution, and dried over anhydrous Na_2SO_4. After concentration, tetraalkyl spermine was obtained as a colorless oil. The material was fully characterized by IR and NMR.

Tetra alkyl spermine (2.5 mmole) was dissolved in 60 mL of methyl iodide. The mixture was kept in the dark without stirring for 4.5 hours. After removal of the methyl iodide on a rotary evaporator, the residue was dissolved in 300 mL of methylene chloride, and extracted twice with 150 mL of 10% $NaHCO_3$ solution. After concentration, the desired tetramethyl tetraalkyl spermine was obtained as a light brown solid. Characterization was done by IR, NMR and FAB-MS.

Example 2 In Vivo Transfection Studies

I. Materials and Methods

Plasmid DNA Vectors

Gene transfer studies were carried out with plasmid DNA encoding the firefly *Photinus pyralis* luciferase (luc) reporter gene under the control of the immediate early cytomegaloviral promoter (CMV). This vector, which is known as pCMV-luc, has been described previously (Davis, H.L., *et al.*, *Human Gene Ther.* 4:151-159 (1993)). The DNA was purified on Qiagen anion-exchange chromatography columns (Qiagen GmbH, Hilden, Germany) and resuspended in sterile saline (0.15 M NaCl, Sigma). The concentration of DNA was calculated based on absorbance of ultraviolet light (OD 260) with final concentrations usually being 5-10 mg/ml. DNA solutions were stored at -20°C until required for administration. DNA was administered either as pure plasmid DNA in saline ("naked" DNA) or formulated with the cationic lipids described below.

DNA/lipid formulations

The lipids were formulated into a liposome using the reverse evaporation method. A known amount of cationic lipid was mixed with the neutral co-lipid in a 2L round bottom flask to obtain the desired molar ratio of cationic:neutral co-lipid. Methylene chloride or 10% methanol in methylene chloride is added to make a 10-30 mg/mL solution. GIBCO water is added to give approximately a 1 or 2 mg/mL solution. The mixture is shaken 3-5 minutes and let to stand for approximately 3 minutes. The solution was subjected to rotary evaporation to remove the organics. The volume was adjusted to obtain the desired concentration (1 or 2 mg/mL). The liposome solution was then flushed with nitrogen and stored at 4°C under nitrogen.

The lipid-formulated DNA was administered in a total volume of 150 µl that contained 1-100 µg DNA and an amount of lipid that was from 0.2 to 5 times

by weight the amount of DNA. Prior to formulation with DNA, the solutions of lipids were thoroughly mixed by vortexing.

For each solution to be administered to a group of mice, the volumes of stock DNA solution, stock lipid solution, and diluent (0.15 M NaCl) needed were calculated. The diluent was first added to the lipid and vortexed briefly, then the DNA was added and the mixture was briefly vortexed again. The final mixture was left, undisturbed on ice for 30 minutes to allow DNA/liposome complex formation. Immediately before administration, the DNA/lipid mixture was mixed gently by tapping on the side of Eppendorf tube.

To determine if all the DNA was complexed with the lipid, mixtures were run on a 0.5% agarose gel and stained with 0.04 μg/ml ethidium bromide. The presence of free plasmid was detected as a band at the expected distance whereas DNA-lipid complexes failed to enter the gel.

In vivo gene transfer

Animals. All experiments were carried out using female BALB/c mice (Charles River, Montreal, QC) aged 6-8 weeks with 10 mice per experimental or control group. Each animal received naked or lipid-formulated DNA in a total volume of 150 μl, although this contained different doses of DNA and different relative proportions of DNA and lipid. The DNA was administered to the respiratory system of the mice via intranasal (IN) or intratracheal (IT) routes.

IN instillation. Mice were lightly anaesthetized with Halothane® (Halocarbon Laboratories, River Edge, NJ) and held on their back with their head hanging down. The DNA solution was then instilled bilaterally into the nasal cavity using a gel-loading tip and a Gilson pipette. The tip was inserted a few mm into the nasal cavity and each nostril was instilled 3 times at 15 minute intervals with 25 μl of DNA solution (for a total volume of 150 μl/mouse).

IN inhalation. The DNA was delivered by a pipette tip, but this was not placed inside the nasal cavity. Rather, the DNA solution was deposited as droplets applied bilaterally directly over the external nares of mice under

Halothane anesthesia. A volume of 25 µl was placed over each nostril 3 separate times (for a total volume of 150 µl) with 15 minute intervals between administrations.

IT injection. Mice fully anaesthetized with Somnotol® (75 mg/kg IP; MTC Pharmaceuticals, Cambridge, ON) had their trachea exposed through an anterior midline incision. The DNA solution (150 µl) was then injected through the anterior wall of the trachea using a 0.3 cc insulin syringe with a 29-gauge needle attached (Becton Dickenson, Franklin Lakes, New Jersey, USA). This was given either as a single injection or as three injections of 50 µl each, with 15 minute intervals between administrations. The incision was sutured and the mouse was placed in an incubator until fully recovered from the anesthetic (approximately 45 min).

IT cannulation. Mice under Somnotol® anesthesia had their trachea exposed via an anterior midline incision, and this was used to visualize the insertion of the cannula into the trachea. A 20-gauge olive tip steel feeding tube (Fine Science Tools Inc., North Vancouver, BC, Canada) attached to a 1 c.c. tuberculin syringe (Becton Dickenson, Franklin Lakes, New Jersey, USA) was passed through the oral cavity and into the trachea. The DNA solution (150 µl) was then slowly injected directly into the lungs.

Evaluation of reporter gene expression

Mice were killed by cervical dislocation under Halothane anesthesia at various times from 6 hr to 19 days after gene transfer. The lungs were dissected free, homogenized and assayed for luciferase reporter gene activity using the Promega Luciferase Assay System (Madison, WI, USA) according to a method previously described for muscle (Davis, H.L., *et al.*, *Human Gene Ther.* 4:151-159 (1993)) except that the lungs were homogenized in 350 µl of 1x lysis buffer. Measurements were taken with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA, USA) and data was expressed as total relative light units/sec (RLU/sec).

Experimental groups

Effect of DNA:lipid ratio. A total of 25 μg pCMV-luc DNA mixed with lipid 301 at DNA:lipid (w/w) ratios of 1:0 (pure DNA), 5:1, 3:1, 2:1, 1:1, 1:2, 1:3 and 1:5 was administered to mice by the IN inhalation method. Luciferase activity in the lungs was determined 48 hr later.

DNA dose. Mice received a total of 1, 10, 25, 50 or 100 μg of pCMV-luc DNA, formulated with lipid 301 at a 1:1 DNA:lipid ratio (w/w). This was given by IN inhalation in a total volume of 150 μl. Lungs were assayed for luciferase activity at 48 hr.

Longevity of reporter gene expression. A total of 100 μg pCMV-luc DNA formulated with lipid 301 at a 1:1 DNA:lipid (w/w) ratio was administered by IN inhalation to each mouse. Lungs were removed from mice killed at 6 hr, 1, 2, 3, 4, 5, 7, 9, 14 or 19 days after gene transfer and assayed for luciferase activity.

Method of DNA administration. A total of 100 μg pCMV-luc DNA formulated with lipid 301 at a 1:1 DNA:lipid (w/w) ratio was administered by each of the four methods described above (IN instillation, IN inhalation, IT injection, IT cannulation). Luciferase activity in the lungs was assayed 24, 48 or 72 hr later.

Lipid composition. A total of 100 μg pCMV-luc DNA was formulated with each of the 14 different lipids (see Table 2) at a 1:1 DNA:lipid (w/w) ratio. These solutions were administered to mice by IN inhalation and luciferase activity was detected in the lungs 72 hr later.

II. Results

Effect of DNA:lipid ratio

DNA formulated with lipid 301 at different DNA: lipid w/w ratios and administered to mice by IN inhalation showed that the ratio has an effect on transfection efficiency. The highest mean luciferase expression was obtained

using a DNA:lipid ratio of 1:2, although ratios from 1:1 to 1:3 all gave relatively high luciferase activity (FIG. 1).

Visualization of complexed and free plasmid DNA on an agarose gel revealed that DNA was fully complexed to lipid 301 at about a DNA:lipid ratio of 1:2. Thus optimal gene transfer was attained when the lipid was complexed with the maximum amount of DNA possible. For a given dose of DNA (in this case 25 μg), the efficiency of gene transfer into the lung decreased proportionate to the amount of free DNA, with the lowest luciferase activity being detected with pure plasmid DNA. Efficiency also decreased somewhat with the formulation containing extra lipid, possibly owing to toxicity of the lipid.

In order to attain a high efficiency of gene transfer with the lowest amount of lipid (and thus the least toxicity), all subsequent experiments were carried out using a 1:1 DNA:lipid (w/w) ratio.

Effect of DNA dose

A linear dose-response was detected for luciferase activity in the lung 48 hr after administration of amounts from 1 to 100 μg of pCMV-luc DNA (mixed 1:1 w/w with lipid 301) by IN inhalation (coefficient of correlation = 0.98) (FIG. 2). Thus it appears that the uptake of DNA is limited not by saturation with DNA but rather by the volume than can be given without causing undue stress to the animal.

A dose of 100 μg of DNA was used in all subsequent experiments.

Longevity of reporter gene expression

Low levels of luciferase expression could be detected as little as 6 hours after IN inhalation of 100 μg pCMV-luc (mixed 1:1 w/w with lipid 301). The reporter gene activity steadily increases up until 4 days, then declines rapidly between 4 and 5 days and reaches very low levels by 9 days (FIG. 3).

Thus foreign gene expression commences quickly, but gene product continues to accumulate over several days. The rapid drop-off of activity after

4 days is likely due to cell turn-over in the lung. The very low activity that remains more or less constant from 9 to 19 days indicates that a few cells with slower turnover may be transfected. These cells might be of a different type than the predominant epithelial cell.

Method of DNA administration

Administration of pCMV-luc DNA (100 μg) by any of the four routes tested (IN inhalation, IN instillation, IT injection and IT cannulation) results in transfection of cells in the lung by both pure plasmid DNA and DNA mixed 1:1 (w/w) with lipid 301, however some methods clearly result in more efficient transfection than do other methods (FIG. 4).

For any given route, the lipid-formulated DNA is superior to pure plasmid DNA, giving approximately 10-fold higher levels of luciferase reporter gene activity. The two IN routes are basically equivalent to each other, which is not unexpected since both methods deliver the DNA into the nasal cavity, from where a large portion of it reaches the lungs. In contrast, the IT cannulation results in about 10-fold higher reporter gene activity than IT injection. This difference is likely due to leakage from the perforated trachea with the injection method. The cannulated IT route has somewhat higher efficiency of transfection that the two IN routes and the IT injection, however the latter methods result in less variability (coefficient of variation >150% and <100% respectively). Thus even though the highest mean titers were obtained with IT cannulation, the IN inhalation method was chosen for assay of different lipids since it is easy and non-invasive and has less variability.

Effect of lipid composition

The nature of the lipid greatly influences the transfection efficiency in lung tissue. The results obtained with IN (inhalation) delivery of pCMV-luc DNA (100 μg) formulated with the 14 different lipids follow a basically bimodal distribution. With lipids that work well (i.e., 301, 302, 303, 9231 and 9232), the

efficiency of gene transfer, as indicated by mean luciferase activity 72 hr later, is approximately the same (5-10 times higher than with naked DNA), although some lipids gave more variable results than others. On the other hand, lipids that worked poorly (304, 305 [lipofectamine], 201, 202, 203, 204, 205, 206 and lipofectin), all resulted in mean luciferase activities less than that obtained with naked DNA (FIG. 5). All lipids had been given the same 1:1 DNA:lipid (w/w) ratio since it had been verified by agarose gel electrophoresis that they each bound similar quantities of DNA.

III. Discussion

Direct gene transfer of respiratory epithelium

The present study demonstrates that it is possible for epithelial mucosal cells in the lung to take up and express foreign genes after administration of plasmid DNA in naked form or formulated with cationic lipids. Depending on the lipid used, the efficiency of gene transfer with cationic lipid-formulated DNA can be higher then, the same as, or less than that with pure plasmid DNA. The DNA solutions may be administered intranasally (inhaled or instilled) or directly into the lungs via the trachea (injected or cannulated), with all four methods resulting in roughly equivalent efficiencies of transfection. There is a linear dose-response for reporter gene expression up to at least 100 μg plasmid DNA, suggesting that much better transfection efficiency could be attained if there was not a volume limitation.

Naked versus lipid-formation DNA for gene delivery to lungs

Pure plasmid DNA results in an extremely low efficiency of transient transfection of cells *in vitro*, and thus this procedure is usually carried out by formulating the DNA with cationic lipids or by some other method such as calcium phosphate precipitation or electroporation. The situation is somewhat different *in vivo* in that some types of cells, most notably mature muscle fibers,

actually take up and express genes significantly better if given in the form of pure plasmid DNA (Wolff, J.A., *et al.*, *Hum. Mol. Genet. 1*:363-369 (1992)). Nevertheless, gene delivery by most routes other than intramuscular injection, has been demonstrated using liposome-formulated DNA (see Lasic, D.D., and Templeton, N.S., *Adv. Drug Delivery Rev. 20(2-3)*:221-226 (1996)). These routes include IT (Canonico, A.E., *et al.*, *Am. J. Respir. Cell Molec. Med. 10*:24-29 (1994); Li, X.-M., *et al.*, *J. Immunol. 157*:3216-3219 (1996); Yoshimura, K., *et al.*, *Nucleic Acids Res. 20*:3233-3240 (1992)), intra-arterial (Nabel, E.G., *et al.*, *Proc. Natl. Acad. Sci. USA 90*:10759-10763 (1993)), intravenous (Canonico, A.E., *et al.*, *Am. J. Respir. Cell Molec. Med. 10*:24-29 (1994); Yokoyama, M., *et al.*, *FEMS Immunol. Med. Microbiol. 14*:221-230 (1996)), intratumor (Egilmez, N.K., *et al.*, *Biochem. Biophys. Res. Commun. 221*:169-73 (1996); Nabel, E.G., *et al.*, *Proc. Natl. Acad. Sci. USA 90*:11307-11311 (1993)), intracranial (Schwartz, B., *et al.*, *Human Gene Ther 6*:1515-1524 (1995)) and by topical administration to the hair follicle (Li, L., and Hoffman, R.M., *Nature Med. 1*:705-706 (1995)).

Cationic lipids form complexes with plasmid DNA via ionic interactions and are thought to bring about gene transfer by attaching to cell surfaces where they can either fuse directly or be endocytosed with subsequent intracellular fusion with endosomal membranes. As the lipid diffuses into the membrane, the lipid-DNA ionic interactions are disrupted, thereby releasing the DNA into the cytoplasm (Felgner et al., *Ann. N.Y. Acad. Sci. 772*:126-139 (1995)). Liposomes may also help improve the efficiency of transfection owing to increased retention time and slower degradation of the complexed DNA.

There has been conflicting reports as to the relative efficiencies of pure plasmid DNA and liposome-formulated DNA for the direct gene transfer in the lung. Some investigators have reported that liposome-formulated but not naked DNA transfects lung tissue (Hazinski, T.A., *et al.*, *Am. J. Respir. Cell Molec. Med. 4*:206-209 (1991); Stribling, R.E., et al., *Proc. Natl. Acad. Sci. USA 89*:11277-11281 (1992)), whereas some groups found measurable expression

following transfection with naked DNA which could be enhanced by associating the DNA with lipid (Wheeler, C.J., *et al.*, *Proc. Natl. Acad. Sci. USA 93*:11454-11459 (1996); Yoshimura, K., *et al.*, *Nucleic Acids Res. 20*:3233-3240 (1992)). This contrasts with other investigators who suggested that the similar results they obtained with lipid-associated DNA and DNA alone were due to solely to the DNA as it disassociated from the lipid in the passageways of the lungs (Meyer, K.B., *et al.*, *Gene Ther. 2*:450-460 (1995); Tsan, M.-F., *et al.*, *Am J. Physiol. 268*:L1052-L1056 (1995)). Many other studies on delivery of lipid-formulated DNA to the respiratory epithelium have failed to include pure plasmid DNA as a control.

This is the most extensive and comprehensive study to date to compare pure plasmid DNA and lipid-formulated DNA for direct gene transfer into the lung. Owing to the evaluation of a wide variety of lipids, it can now be seen clearly why such discrepant results have been reported heretofore in the literature. Of the 14 lipids tested herein, five resulted in transfection efficiencies greatly superior to that with pure plasmid DNA, two were about the same and seven gave worse results. Thus, these results confirm that pure plasmid DNA can transfect cells in lung tissue and that this can be improved upon substantially by formulating the DNA with some, but not all, cationic lipids.

Experimental models to evaluate respiratory gene transfer

It is clear that the DNA can be delivered to the lung by a wide variety of techniques including IN inhalation or instillation, or IT injection or cannulation. The highest mean reporter gene activity (but also the most variable) is obtained with IT cannulation, which delivers all of the DNA directly to the lungs, and the lowest with IT injection, which appears to result in losses of DNA through the punctured trachea. The two IN methods were equivalent to each other and although the DNA must first pass through the nose and throat, where some is most certainly swallowed, the efficiency of lung transfection was only slightly lower and much less variable than that with IT cannulation. This, in addition to

the fact that IN administration is easy and quick to perform and non-invasive, makes IN the preferred method for animal studies. Of course, with IN gene delivery, it is likely that respiratory epithelial cells outside of the lung (e.g., in the nasal cavity) will also be transfected, and in a DNA vaccine model, this could give different results than those that would be obtained with IT delivery directly into the lungs. The length of time over which the DNA is administered does not appear to be important as similar results were obtained with IT (injection) delivery of a single bolus or with periodic delivery of DNA over a period of 45 minutes.

These results indicate the importance of including pure plasmid DNA as a control. This is necessary to determine whether a new lipid being tested actually improves the efficiency of transfection over than with naked DNA. Half of the lipids tested, including the two commercially available lipids lipofectin and lipofectamine (lipid 305), resulted in luciferase activity lower than that obtained with pure plasmid DNA. It was also found that different lipids have different capacities for complexing with DNA and the best results are obtained when the lipid is mixed with the maximum amount of DNA it can complex, but not more. Thus, it is important to determine the optimal DNA:lipid ratio by checking for non-associated DNA with gel electrophoresis. As well, it is necessary to be aware of the kinetics of gene expression as there is a rather brief period of peak expression.

Potential of respiratory gene transfer for clinical application

Based on results in numerous animal models, DNA vaccines need to transfect only relatively few cells in order to induce potent immune responses. As such, the level of reporter gene activity in lungs of mice after direct introduction of either pure plasmid DNA or liposome-formulated DNA will be sufficient to induce immune responses to an antigenic protein. However, most previous studies on DNA vaccines have involved transfection of cells in muscle

tissue, the epidermis or dermis (Davis, H.L., and Brazolot Millan, C.L., *Blood Cell Biochem.* (in press, 1997); Donnelly, J.J., *et al.*, *Life Sci. 60*:163-172 (1996)).

DNA-based immunization via the mucosal epithelium, e.g. the respiratory system, is an easy, non-invasive method to immunize individuals without the use of trained medical personnel. The possibility to carry out mucosal immunization is highly desirable since it triggers both a mucosal and a systemic response, in contrast to systemic immunization which induces solely systemic immunity. Furthermore, immunization at one mucosal surface has been shown to induce immunity at distant mucosal sites. Mucosal immunity is important to prevent pathogen entry at mucosal surfaces, whereas systemic immunity can only deal with pathogens once they have entered the body.

In contrast to the use of DNA for immunization, therapeutic gene transfer to the respiratory system for therapeutic purposes requires a relatively large number of cells to be transfected. This is especially true for treatment of a disease such as CF where all epithelial cells are missing the normal gene product. Even for delivery of other polypeptides such as cytokines, it is necessary to transfect a large number of cells in order to attain physiological levels systemically. In the present experiments, there was a linear dose-response in the mice that showed, at least from 1-100 μg of DNA, no evidence of saturation. In the mouse model used in the present study, the upper dose of DNA was limited by concentration of reagents available and thus volumes. Much higher volumes of more concentrated DNA solution will allow higher transfection efficiency in humans.

Of obvious concern for clinical application is the brief period of gene expression. Luciferase reporter gene expression in the lung peaked at about 4 days and then fell off rapidly between 5 and 9 days, similar to as has been reported by other investigators (Meyer, K.B., *et al.*, *Gene Ther. 2*:450-460 (1995); Wheeler, C.J., *et al.*, *Proc. Natl. Acad. Sci. USA 93*:11454-11459 (1996)). This is not likely to be due to immune responses against the luciferase-expressing cells or to promoter suppression since introduction of the same pCMV-luc DNA into

mouse muscle fibers, which are post-mitotic, results in high levels of expression for at least 60 days (Davis, H.L., and Brazolot Millan, C.L., *Blood Cell Biochem.* (in press, 1997)). Rather, the rapid loss of activity in the lung is likely due to cell turnover. While the period of foreign gene expression in the lung is likely of sufficient duration for induction of immune responses, and may even be useful for therapies such as short-term IFN-γ delivery to treat asthma, it may be inadequate to provide long term beneficial effects for a disease like cystic fibrosis. In this case, it will be necessary to administer the gene as frequently as once weekly. This argues for the use of plasmid DNA over viral vectors which induce immune responses against themselves and thus preclude the subsequent use of the same vector.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following Claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those in the art to which the invention pertains. All publications, patents and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference in their entirety.

What Is Claimed Is:

1. A method for transfecting mucosal epithelial cells of a mammal with a polynucleotide molecule, comprising:

(a) mixing at least one cationic lipid with a polynucleotide molecule, wherein the amount of lipid is sufficient to complex substantially said polynucleotide molecule, thereby forming a cationic lipid-polynucleotide complex; and

(b) contacting the lipid-polynucleotide complex with the mucosal epithelia of said mammal, whereby said cells become transfected.

2. The method of claim 1, wherein the ratio of polynucleotide to lipid is about 1:1 to 1:3 (w/w).

3. The method of claim 1, wherein the ratio of polynucleotide to lipid is about 1:2.

4. The method of claim 1, wherein said at least one cationic lipid is selected from the group consisting of:

(a) a TMTPS:DOPE mixture;

(b) a TMTPS:C-16dl-PE mixture;

(c) a DMRIE:cholesterol mixture;

(d) a TMTOS:DOPE mixture; and

(e) a TMTOS:C-16dl-PE mixture.

5. The method of claim 1, wherein said at least one cationic lipid is selected from the group consisting of:

(a) a TMTPS:DOPE mixture (ratio about 1:1.5 (w/w));

(b) a TMTPS:C-16dl-PE mixture (ratio about 1:1.5 (w/w));

(c) a DMRIE:cholesterol mixture (ratio about 1:1 (w/w));

(d) a TMTOS:DOPE mixture (ratio about 1:1.5 (w/w)); and

(e) a TMTOS:C-16dl-PE mixture (ratio about 1:1.5 (w/w)).

6. The method of claim 1, wherein said polynucleotide codes for a protein.

7. The method of claim 1, wherein said polynucleotide codes for an antigen capable of raising an immune response in said mammal.

8. The method of claim 1, wherein said polynucleotide is an antisense oligonucleotide.

9. The method of claim 1, wherein said polynucleotide molecule is effective for gene therapy.

10. The method of claim 1, wherein said mucosal cells are transfected *in vivo*.

11. A method for generating an immune response against an infectious disease in an animal, comprising the steps of:

(a) mixing at least one cationic lipid with a polynucleotide coding for an antigen capable of generating an immune response in said mammal, wherein the amount of lipid is sufficient to complex substantially said DNA molecule, thereby forming a cationic lipid-polynucleotide complex; and

(b) administering the lipid-polynucleotide complex to the mucosal epithelia of said mammal;

whereby an immune response to the infectious disease is generated.

12. The method of claim 11, wherein the ratio of polynucleotide to lipid is about 1:1 to 1:3 (w/w).

13. The method of claim 11, wherein the ratio of polynucleotide to lipid is about 1:2.

14. The method of claim 11, wherein said cationic lipid is selected from the group consisting of:

(a) a TMTPS:DOPE mixture;

(b) a TMTPS:C-16dl-PE mixture;

(c) a DMRIE:cholesterol mixture;

(d) a TMTOS:DOPE mixture; and

(e) a TMTOS:C-16dl-PE mixture.

15. The method of claim 11, wherein said at least one cationic lipid is selected from the group consisting of:

(a) a TMTPS:DOPE mixture (ratio about 1:1.5 (w/w));

(b) a TMTPS:C-16dl-PE mixture (ratio about 1:1.5 (w/w));

(c) a DMRIE:cholesterol mixture (ratio about 1:1 (w/w));

(d) a TMTOS:DOPE mixture (ratio about 1:1.5 (w/w)); and

(e) a TMTOS:C-16dl-PE mixture (ratio about 1:1.5 (w/w)).

16. A method for producing polyclonal antibodies, comprising (a) mixing at least one cationic lipid with a polynucleotide coding for an antigen capable of generating an immune response in a mammal, wherein the amount of lipid is sufficient to complex substantially said DNA molecule, thereby forming a cationic lipid-polynucleotide complex; and

(b) administering the lipid-polynucleotide complex to the mammal, whereby an immune response to the antigen is generated.

17. The method of claim 16, further comprising

(c) isolating the polyclonal antibodies from the immunized mammal.

18. A method for producing monoclonal antibodies, comprising:

(a) mixing at least one cationic lipid with a polynucleotide coding for an antigen capable of raising an immune response in a mouse, wherein the amount of lipid is sufficient to complex substantially said DNA molecule, thereby forming a cationic lipid-polynucleotide complex;

(b) administering the lipid-polynucleotide complex to at least one mammal;

(c) detecting whether an immune response to said antigen has occurred;

(d) removing B-lymphocytes from the mammal;

(e) fusing the B-lymphocytes with myeloma cells, thereby producing hybridomas;

(f) cloning the hybridomas;

(g) selecting positive clones which produce anti-immunogen antibody;

(h) culturing the anti-immunogen antibody-producing clones; and

(i) isolating anti-immunogen antibodies from the cultures.

19. The method of claim 18, wherein said mammal is a mouse.

20. A cationic lipid mixture selected from the group consisting of:

(a) a TMTPS:DOPE mixture;

(b) a TMTPS:C-16dl-PE mixture;

(c) a DMRIE:cholesterol mixture;

(d) a TMTOS:DOPE mixture; and

(e) a TMTOS:C-16dl-PE mixture.

21. The mixture of claim 20, selected from the group consisting of:

(a) a TMTPS:DOPE mixture (ratio about 1:1.5 (w/w));

(b) a TMTPS:C-16dl-PE mixture (ratio about 1:1.5 (w/w));

(c) a DMRIE:cholesterol mixture (ratio about 1:1 (w/w));

(d) a TMTOS:DOPE mixture (ratio about 1:1.5 (w/w)); and

(e) a TMTOS:C-16dl-PE mixture (ratio about 1:1.5 (w/w)).

22. A complex between a polynucleotide molecule and a cationic lipid mixture selected from the group consisting of:

(a) a TMTPS:DOPE mixture;

(b) a TMTPS:C-16dl-PE mixture;

(c) a DMRIE:cholesterol mixture;

(d) a TMTOS:DOPE mixture; and

(e) a TMTOS:C-16dl-PE mixture.

23. The complex of claim 22, wherein said lipid mixture is selected from the group consisting of:

(a) a TMTPS:DOPE mixture (ratio about 1:1.5 (w/w));

(b) a TMTPS:C-16dl-PE mixture (ratio about 1:1.5 (w/w));

(c) a DMRIE:cholesterol mixture (ratio about 1:1 (w/w));

(d) a TMTOS:DOPE mixture (ratio about 1:1.5 (w/w)); and

(e) a TMTOS:C-16dl-PE mixture (ratio about 1:1.5 (w/w)).

24. The complex of claim 22, wherein the ratio of polynucleotide to lipid mixture is about 1:1 to 1:3 (w/w).

25. The complex of claim 2, wherein the ratio of polynucleotide to lipid is about 1:2.

26. A method of obtaining a polynucleotide/cationic lipid complex, comprising admixing a polynucleotide with at least one cationic lipid in an amount sufficient to complex substantially said polynucleotide molecule, thereby forming a cationic lipid-polynucleotide complex.

27. A polynucleotide/cationic lipid complex obtained by the method of claim 26.

28. The polynucleotide/cationic lipid complex of claim 27, wherein said at least one cationic lipid is selected from the group consisting of:

(a) a TMTPS:DOPE mixture;

(b) a TMTPS:C-16dl-PE mixture;

(c) a DMRIE:cholesterol mixture;

(d) a TMTOS:DOPE mixture; and

(e) a TMTOS:C-16dl-PE mixture.

29. The polynucleotide/cationic lipid complex of claim 27, wherein said at least one cationic lipid is selected from the group consisting of:

(a) a TMTPS:DOPE mixture (ratio about 1:1.5 (w/w));

(b) a TMTPS:C-16dl-PE mixture (ratio about 1:1.5 (w/w));

(c) a DMRIE:cholesterol mixture (ratio about 1:1 (w/w));

(d) a TMTOS:DOPE mixture (ratio about 1:1.5 (w/w)); and

(e) a TMTOS:C-16dl-PE mixture (ratio about 1:1.5 (w/w)).

30. A lipid selected from the group consisting of C16-PE and C16-dl-PE.

31. A complex, comprising the lipid of claim 30 in admixture together with at least one cationic lipid complexed to a polynucleotide molecule.

32. The complex of claim 31, in the form of a liposome.

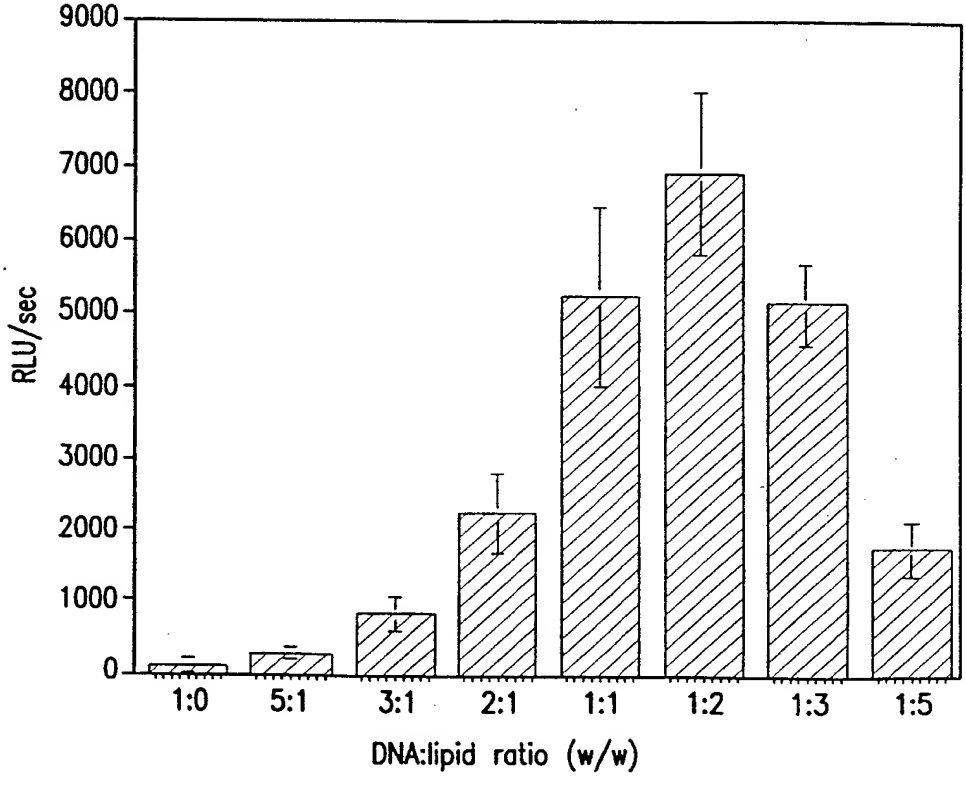

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
RLU/sec
1:0
5:1
3:1
2:1
1:1
1:2
1:3
1:5
DNA:lipid ratio (w/w)

FIG. 1

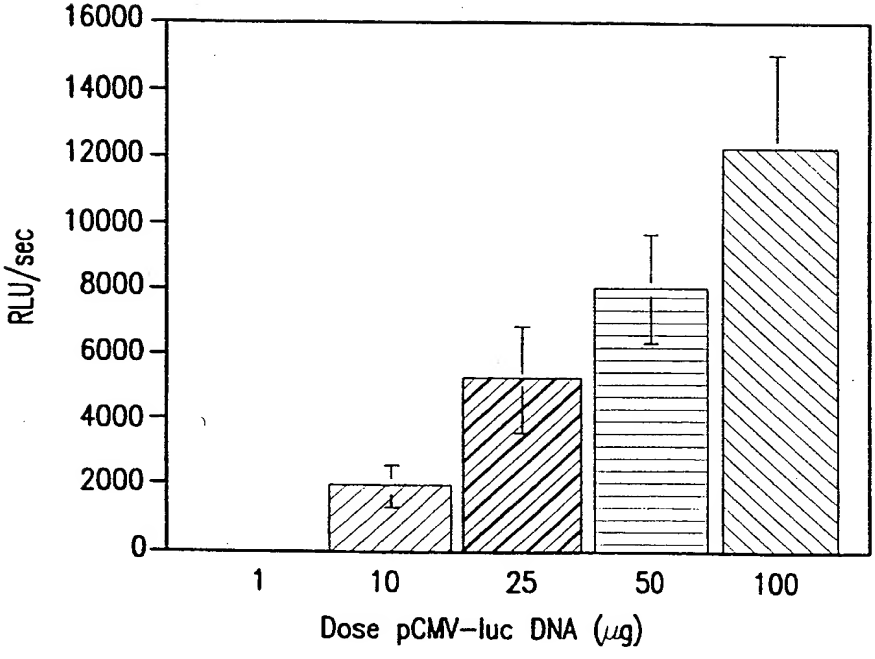


FIG.2

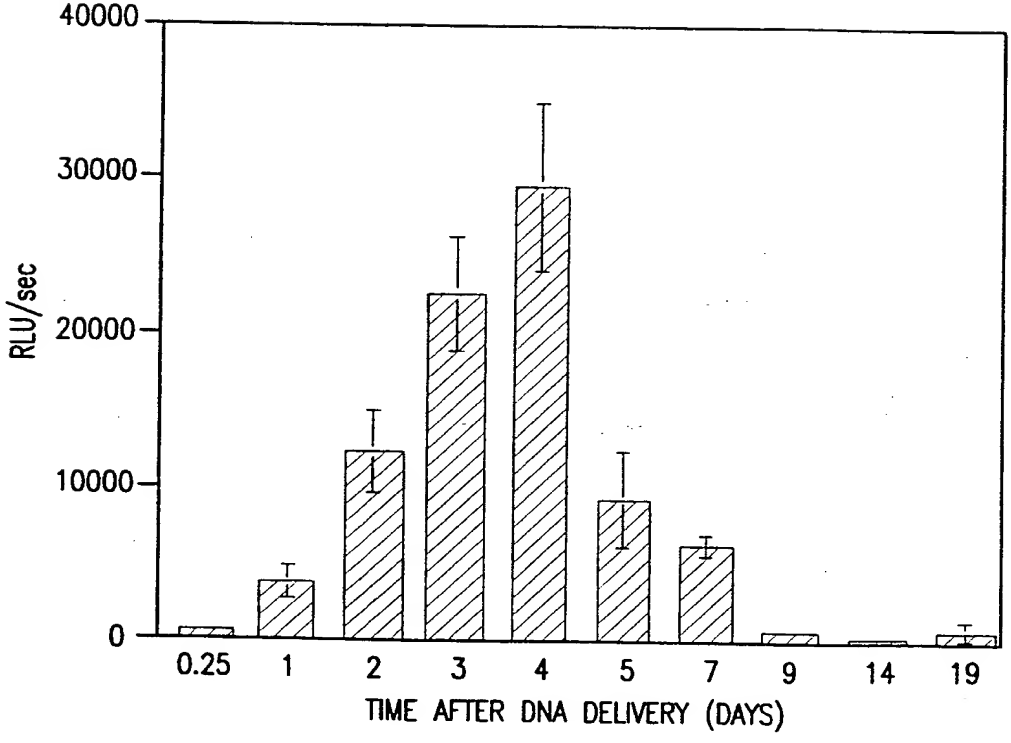


FIG.3

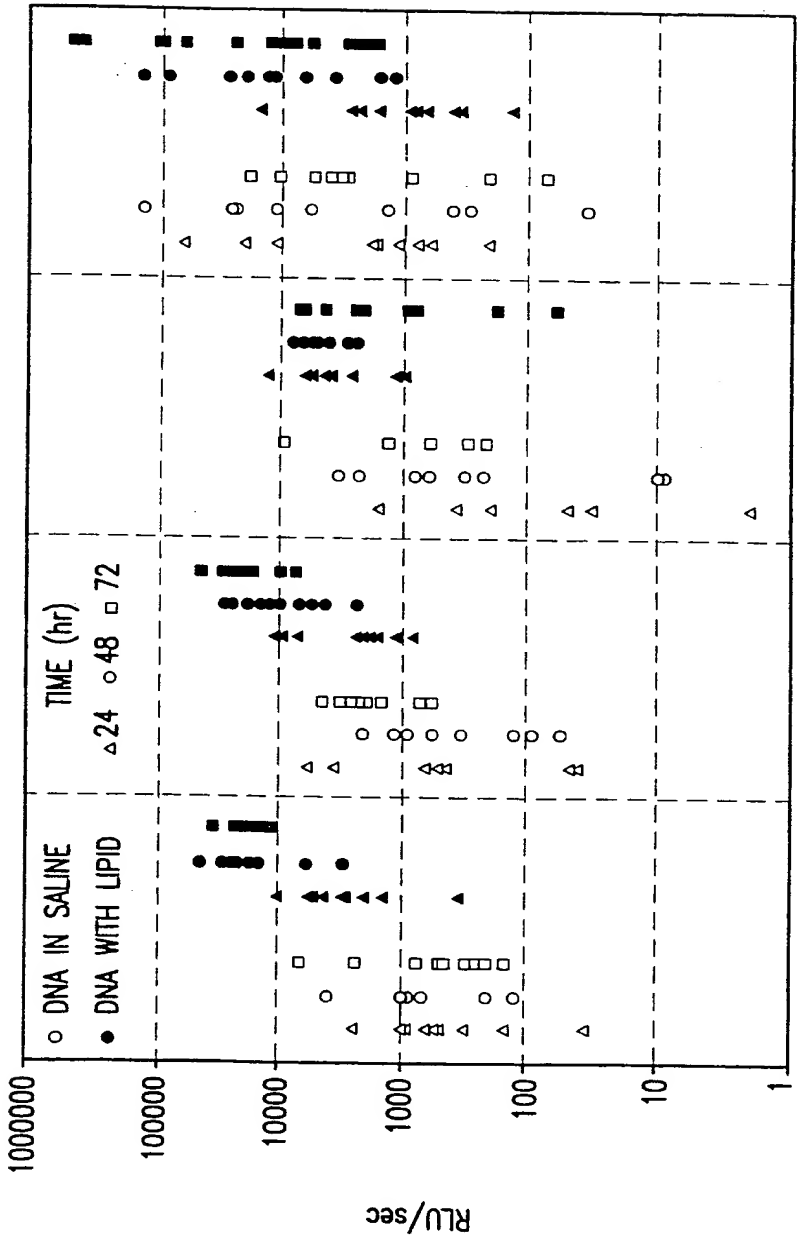
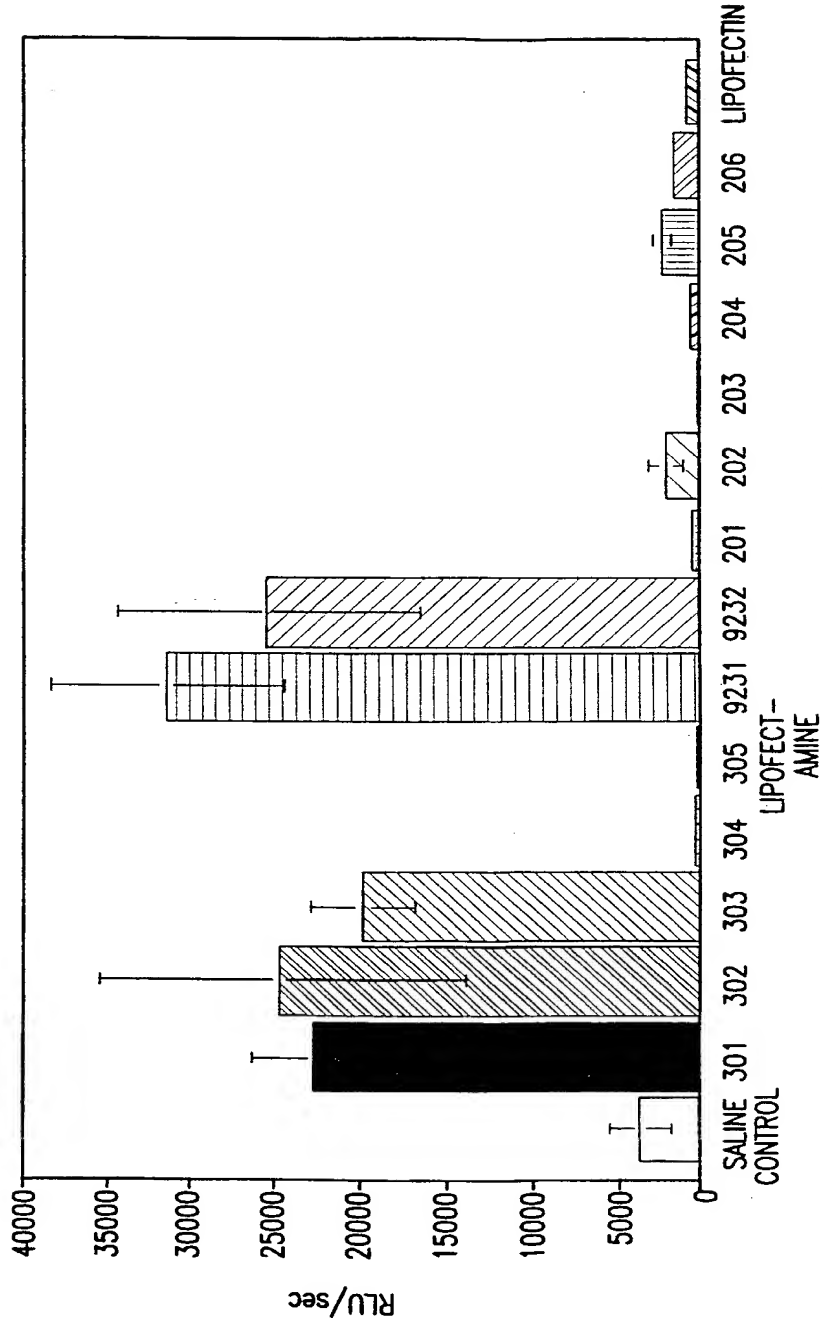


FIG.4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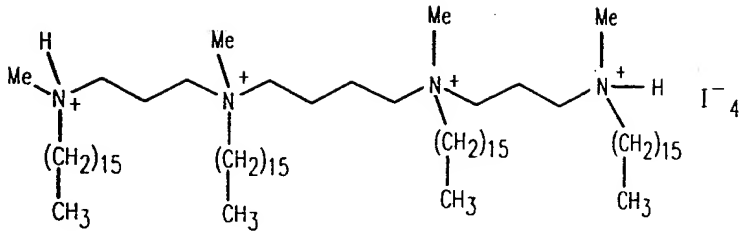
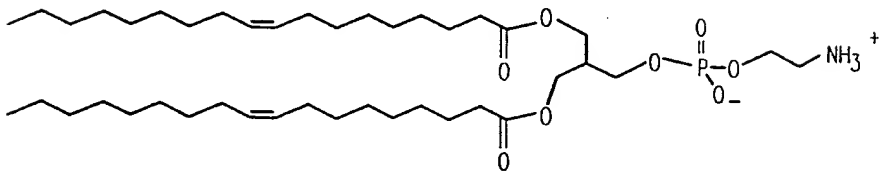
30000
25000
20000
15000
10000
5000
0
SALINE CONTROL
301
302
303
304
305
LIPOFECT-AMINE
9231
9232
201
202
203
204
205
206
LIPOFECTIN

FIG.5



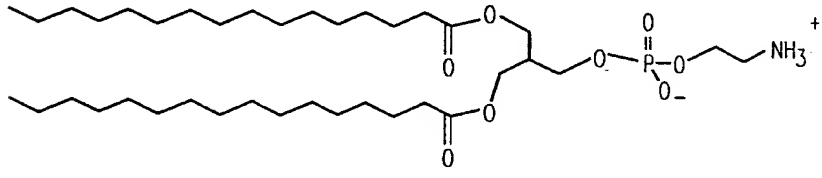
Tetramethyl-tetrapalmityl-spermine (TMTPS)

(I)



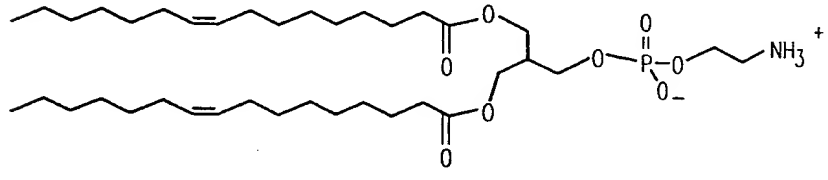
Dioleylphosphatidylethanolamine (DOPE)

II



Dipalmitoylphosphatidylethanolamine (C-16-PE)

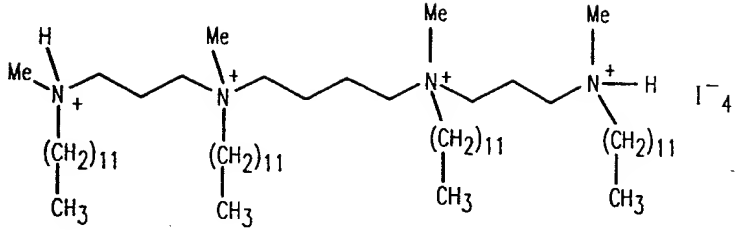
III



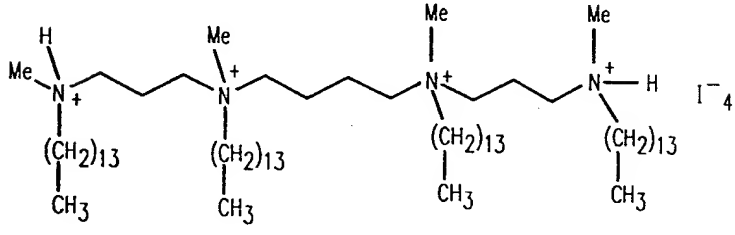
Dipalmitoleoylphosphatidylethanolamine (C-16d1-PE)

IV

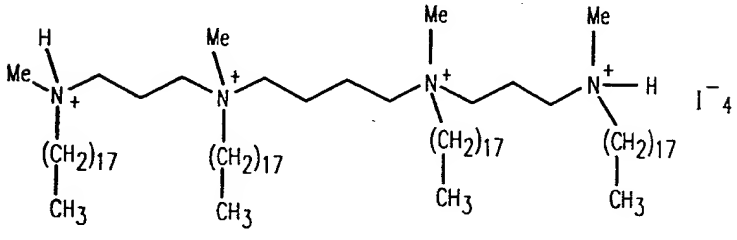
FIG.6A



Tetramethyltetralaureylspermine (TMTLS)
VI

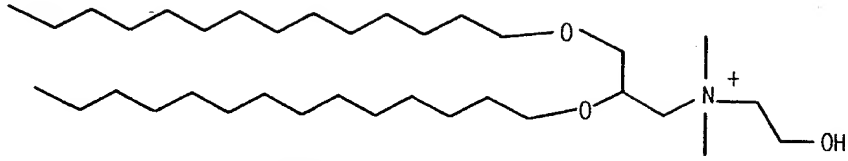


Tetramethyltetramyristylspermine (TMTMS)
VII

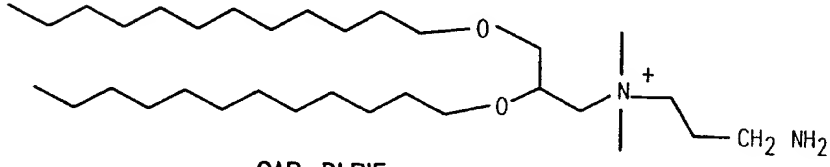


Tetramethyltetrasterylspermine (TMTSS)
VIII

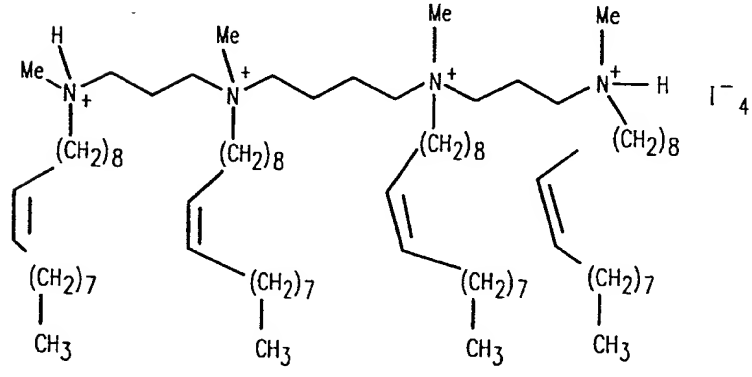
FIG.6B



DMRIE
XXV



GAP–DLRIE
XXVI



Tetramethyltetraoleoylspermine (TMTOS)
XXVII

FIG.6C

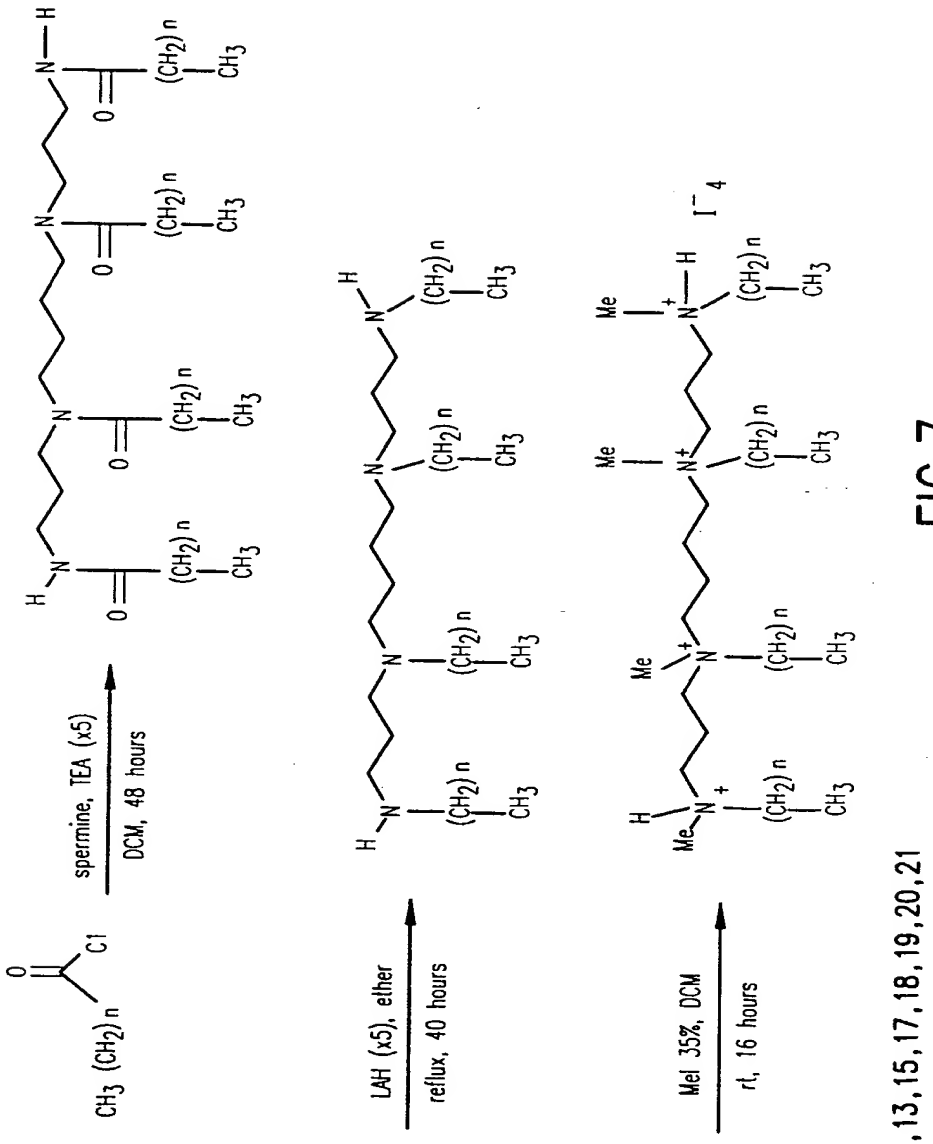


FIG.7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03421

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/63; A01N 43/04; A61K 31/70
US CL :435/ 320.1,.172.3, 69.1, 69.3; 514/44; 424/885, 93.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/ 320.1, 172.3, 69.1, 69.3; 514/44; 424/885, 93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,589,466 A (FELGNER ET AL.) 31 December 1996, see entire document.	1-32
Y	NABEL et al. Direct gene transfer for treatment of human cancer. Annals of the New York Academy of Sciences. November 1995, Vol. 772, pages 227-231, see especially page 229.	1-32
Y	STOPECK et al. Phase I study of direct gene transfer of an allogeneic histocompatibility antigen, HLA-B7, in patients with metastatic melanoma. Journal of Clinical Oncology. January 1997, Vol. 5, No. 1, pages 341-349, see entire document.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
06 MAY 1997

Date of mailing of the international search report
29 MAY 1997

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Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03421

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NABEL et al. Immune response in human melanoma after transfer of an allogeneic class I major histocompatibility complex gene with DNA-liposome complexes. Proceedings of the National Academy of Sciences, USA. December 1996, Vol. 93, pages 15388-15393, see entire document.	1-32
Y	ALBRECHT et al. Cationic lipid mediated transfer of c-abl and bcr antisense oligonucleotides to immature normal myeloid cells: uptake, biological effects and modulation of gene expression. Annals of Hematology. February 1996, Vol. 72, No. 2, pages 73-79, see entire document.	1-5, 8
Y	HARRISON et al. Optimization of gene transfer using cationic lipids in cell lines and primary human $CD4^+$ and $CD34^+$ hematopoietic cells. Biotechniques. 1995, Vol. 19, No. 5, pages 816-820 and 822-823, see entire document.	1-6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03421

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, EMBASE, BIOSIS, CAPLUS, WPIDS, APS
search terms: lipids, cationic lipids, liposomes, lipid-polynucleotide complexes, transfection, gene transfer, gene delivery, antigens, immune response, mucosal epithelium